2H7scFv-Ig cDNA and predicted amino acid sequence:

```
      HindIII          |2H7 VL Leader Peptide→
      ~~~~~~
                     M  D  F   Q  V  Q   I  F  S  F   L  L  I   S  A  S
1     AAGCTTGCCG CCATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                          |2H7 VL→
       V  I  I  A   R  G  Q   I  V  L   S  Q  S  P   A  I  L   S  A  S
61    GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

       F  G  E  K   V  T  M   T  C  R   A  S  S  S   V  S  Y   M  H  W
121   CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                     BamHI
                     ~~~~~~~
       Y  Q  Q  K   P  G  S   S  P  K   P  W  I  Y   A  P  S   N  L  A
181   TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

       S  G  V  P   A  R  F   S  G  S   G  S  G  T   S  Y  S   L  T  I
241   TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

       S  R  V  E   A  E  D   A  A  T   Y  Y  C  Q   Q  W  S   F  N  P
301   AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

                                                  |(Gly4Ser)3 Linker
       P  T  F  G   A  G  T   K  L  E   L  K  G  G   G  G  S   G  G  G
361   CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                          |2H7 VH →
       G  S  G  G   G  G  S   S  Q  A   Y  L  Q  Q   S  G  A   E  L  V
421   GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

       R  P  G  A   S  V  K   M  S  C   K  A  S  G   Y  T  F   T  S  Y
481   AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

       N  M  H  W   V  K  Q   T  P  R   Q  G  L  E   W  I  G   A  I  Y
541   AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

       P  G  N  G   D  T  S   Y  N  Q   K  F  K  G   K  A  T   L  T  V
601   CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

       D  K  S  S   S  T  A   Y  M  Q   L  S  S  L   T  S  E   D  S  A
661   GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

       V  Y  F  C   A  R  V   V  Y  Y   S  N  S  Y   W  Y  F   D  V  W
721   GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

Fig. 1

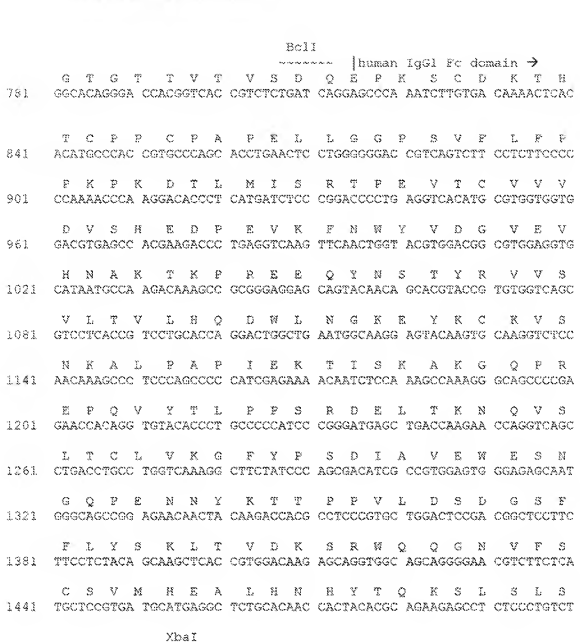
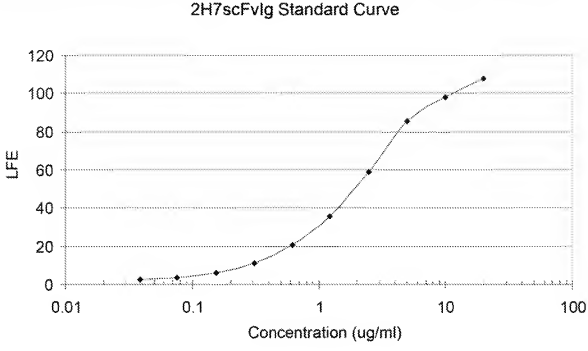


Fig. 1 (continued)

Production Levels of 2H7 scFvIg by Stable CHO Lines



Clone	LFE @ 1:50 Estimated Concentration (µg/ml)
D2	26.156
IIIC6	25.755
IVA3	28.661
Spent bulk	29.664

Fig. 2

SDS-PAGE Analysis of
2H7 scFvIGG1 (SSS-S)H WCH2 WCH3 Protein.

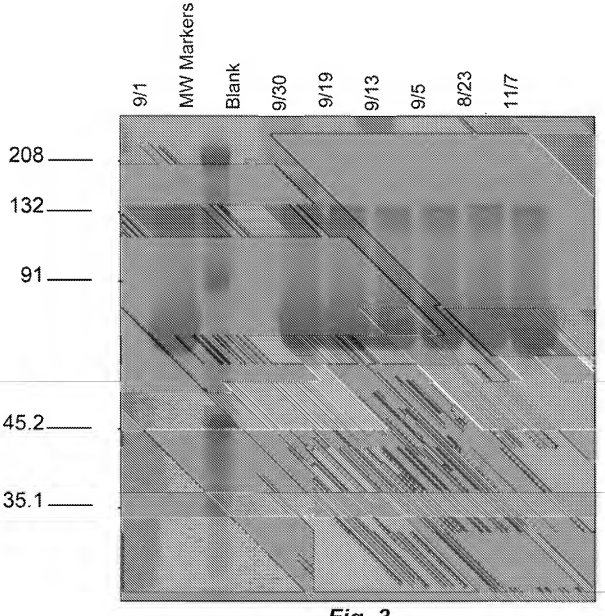


Fig. 3

Complement Mediated B Cell Killing After Binding of CD20-targeted 2H7 scFvIGG1 (SSS-S) H WCKH2 WCKH3:

2H7scFv-Ig Concentration	RAMOS # live cells/total cells		BJAB # live cells/total cells	
20 µg/ml + complement	-	0.16	-	0.07
5 µg/ml + complement	-	0.2	-	N.D.
1.25 µg/ml + complement	-	0.32	-	0.1
Complement alone	-	0.98	-	0.94

*Viability was determined by trypan blue exclusion and is tabulated as the fraction of viable cells out of the total number of cells counted.
**N.D. (not determined).

Fig. 4A

Antibody-dependent cellular cytotoxicity (ADCC) mediated by 2H7scFv-IgG1 (SSS-S)H WCH2 WCH3:

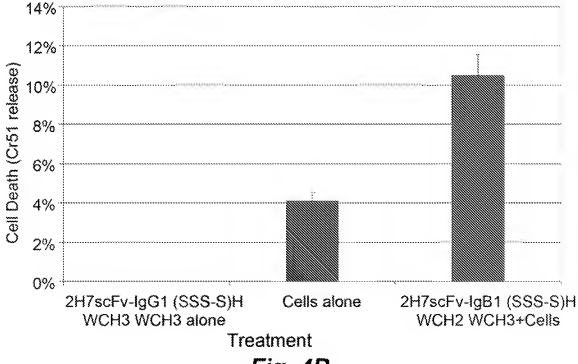


Fig. 4B

Effects of Crosslinking of CD20 and CD40 Cell Surface Receptors on B Cell Proliferation:

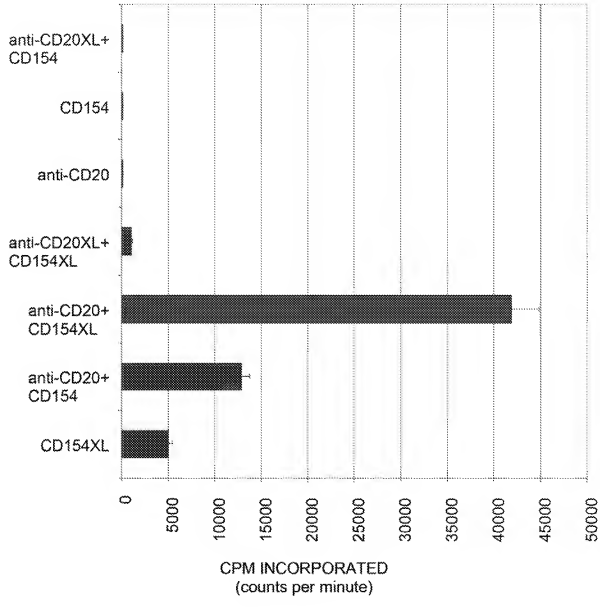


Fig. 5

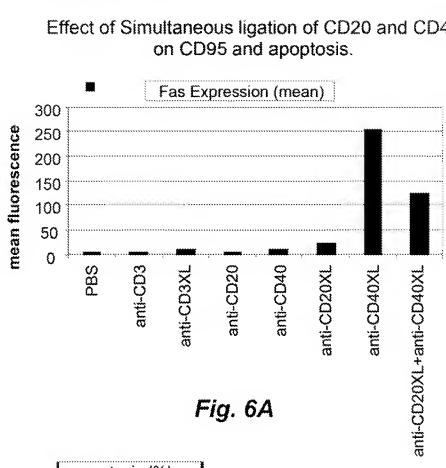

Effect of Simultaneous ligation of CD20 and CD40 on CD95 and apoptosis.
Fas Expression (mean)
mean fluorescence
300
250
200
150
100
50
0
PBS
anti-CD3
anti-CD3XL
anti-CD20
anti-CD40
anti-CD20XL
anti-CD40XL
anti-CD20XL+anti-CD40XL

Fig. 6A

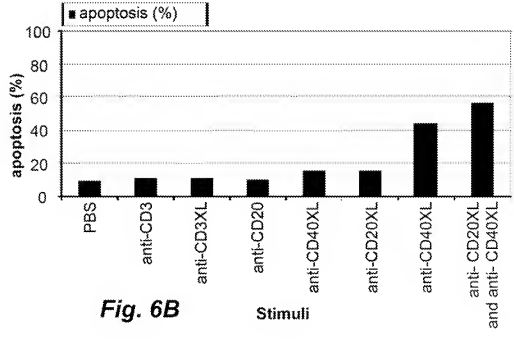

apoptosis (%)
apoptosis (%)
100
80
60
40
20
0
PBS
anti-CD3
anti-CD3XL
anti-CD20
anti-CD40XL
anti-CD20XL
anti-CD40XL
anti- CD20XL and anti- CD40XL
Stimuli

Fig. 6B

2H7-CD154 L2 cDNA and predicted amino acid sequence:

```
      HindIII      NcoI     |2H7 VL Leader Peptide →
      ~~~~~~      ~~~~~~
                            M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A
1     AAGCTTGCCG CC   ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                          |2H7 VL →
       V  I  I  A  R  G  Q  I  V  L  S  Q  S  P  A  I  L  S  A  S
61    GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

       P  G  E  K  V  T  M  T  C  R  A  S  S  S  V  S  Y  M  H  W
121   CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                   BamHI
                   ~~~~~~~
       Y  Q  Q  K  P  G  S  S  P  K  P  W  I  Y  A  P  S  N  L  A
181   TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

       S  G  V  P  A  P  F  S  G  S  G  S  G  T  S  Y  S  L  T  I
241   TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

       S  R  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  F  N  P
301   AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

                                                |(Gly4Ser)3 Linker →
       P  T  F  G  A  G  T  K  L  E  L  K  G  G  G  G  S  G  G  G
361   CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                          |2H7 VH →
       G  S  G  G  G  G  S  S  Q  A  Y  L  Q  Q  S  G  A  E  L  V
421   GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

       R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T  S  Y
481   AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

       N  M  H  W  V  K  Q  T  P  R  Q  G  L  E  W  I  G  A  I  Y
541   AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

       P  G  N  G  D  T  S  Y  N  Q  K  F  K  G  K  A  T  L  T  V
601   CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

       D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  A
661   GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

       V  Y  F  C  A  R  V  V  Y  Y  S  N  S  Y  W  Y  F  D  V  W
721   GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

Fig. 7A

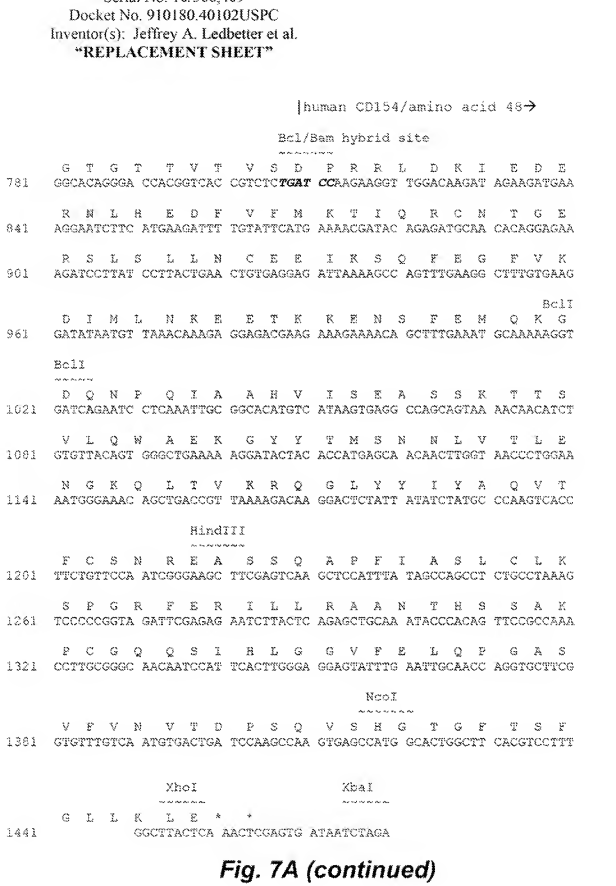


Fig. 7A (continued)

2H7scFv-CD154 S4 cDNA and predicted amino acid sequence:

```
      HindIII      NcoI
      ~~~~~~       ~~~~~~|2H7 VL Leader Peptide→
                           M  D  F   Q  V  Q   I  F  S  F   L  L  I   S  A  S
1     AAGCTTGCCG CC   ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                          |2H7 VL →
       V  I  I  A   R  G  Q   I  V  L   S  Q  S  F   A  I  L   S  A  S
61    GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

       P  G  E  K   V  T  M   T  C  R   A  S  S  S   V  S  Y   M  H  W
121   CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                   BamHI
                   ~~~~~~~
       Y  Q  Q  K   P  G  S   S  P  K   P  W  I  Y   A  P  S   N  L  A
181   TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

       S  G  V  P   A  P  F   S  G  S   G  S  G  T   S  Y  S   L  T  I
241   TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

       S  R  V  E   A  E  D   A  A  T   Y  Y  C  Q   Q  W  S   F  N  P
301   AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

                                                 |(Gly4Ser)3 Linker →
       P  T  F  G   A  G  T   K  L  E   L  K  G  G   G  G  S   G  G  G
361   CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                          |2H7 VH →
       G  S  G  G   G  G  S   S  Q  A   Y  L  Q  Q   S  G  A   E  L  V
421   GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

       R  P  G  A   S  V  K   M  S  C   K  A  S  G   Y  T  F   T  S  Y
481   AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

       N  M  H  W   V  K  Q   T  P  R   Q  G  L  E   W  I  G   A  I  Y
541   AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

       P  G  N  G   D  T  S   Y  N  Q   K  F  K  G   K  A  T   L  T  V
601   CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

       D  K  S  S   S  T  A   Y  M  Q   L  S  S  L   T  S  E   D  S  A
661   GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

       V  Y  F  C   A  R  V   V  Y  Y   S  N  S  Y   W  Y  F   D  V  W
721   GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

Fig. 7B

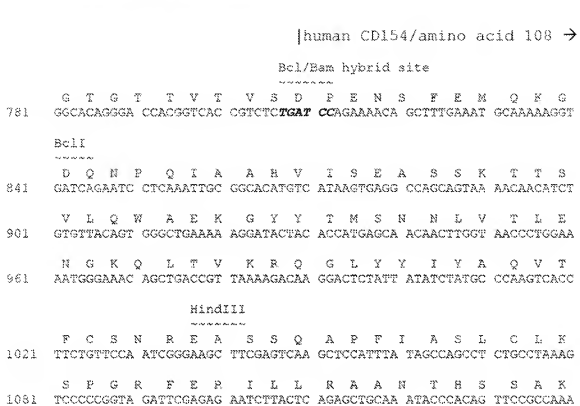
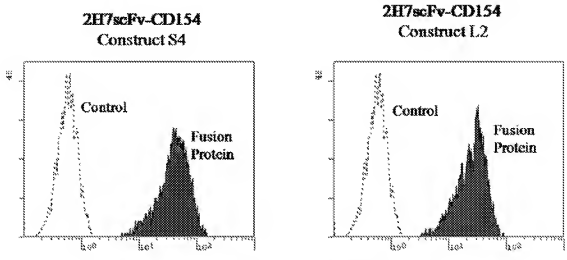


Fig. 7B (continued)

Simultaneous Binding of 2H7scFv-CD154
Fusion Proteins to CD20 and CD40



CD20 CHO cell targets + (control or fusion protein)
+ Biotin-CD40Ig + PE-SA

Fig. 8

Induction of Apoptosis Measured by Binding of Annexin V after incubation with 2H7scFv-CD154

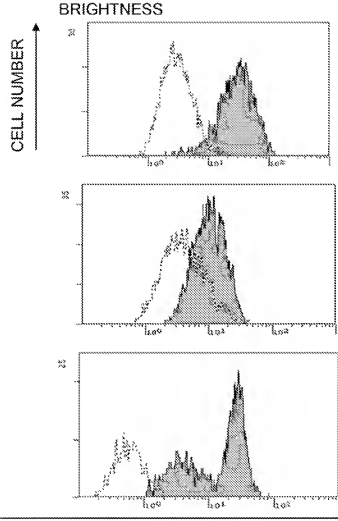


Fig. 9

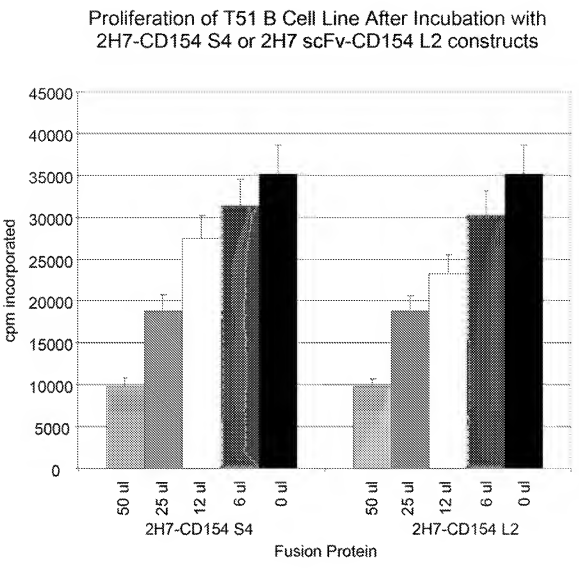

Proliferation of T51 B Cell Line After Incubation with 2H7-CD154 S4 or 2H7 scFv-CD154 L2 constructs
cpm incorporated
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
50 ul
25 ul
12 ul
6 ul
0 ul
2H7-CD154 S4
2H7-CD154 L2
Fusion Protein

Fig. 10

Schematic Representation of 2H7 scFvIg fusion proteins

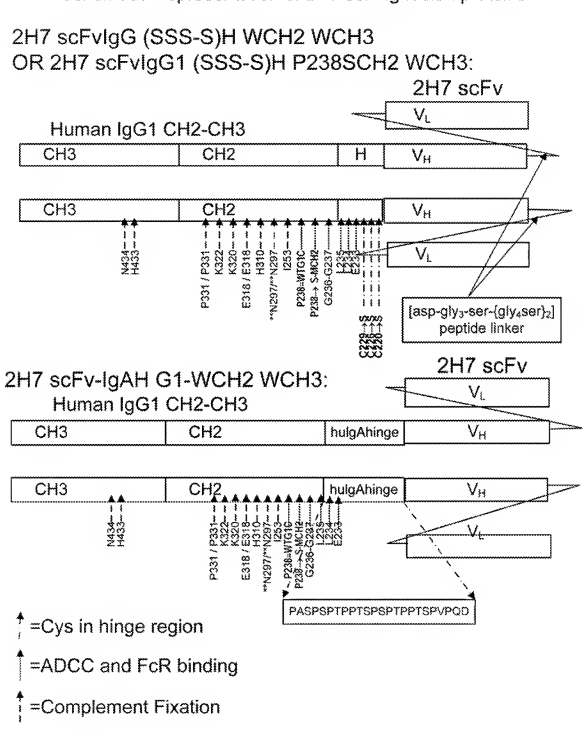


Fig. 11

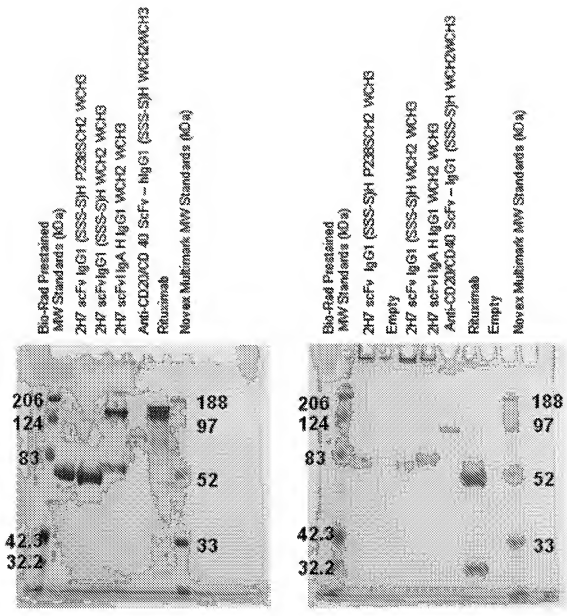

Bio-Rad Prestained MW Standards (kDa)
2H7 scFv IgG1 (SSS-S)H P238SCH2 WCH3
2H7 scFv IgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA H IgG1 WCH2 WCH3
Anti-CD20/CD 40 ScFv – IgG1 (SSS-S)H WCH2WCH3
Rituximab
Novex Multimark MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33
Bio-Rad Prestained MW Standards (kDa)
2H7 scFv IgG1 (SSS-S)H P238SCH2 WCH3
Empty
2H7 scFv IgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA H IgG1 WCH2 WCH3
Anti-CD20/CD40 ScFv – IgG1 (SSS-S)H WCH2WCH3
Rituximab
Empty
Novex Multimark MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33

Fig. 12

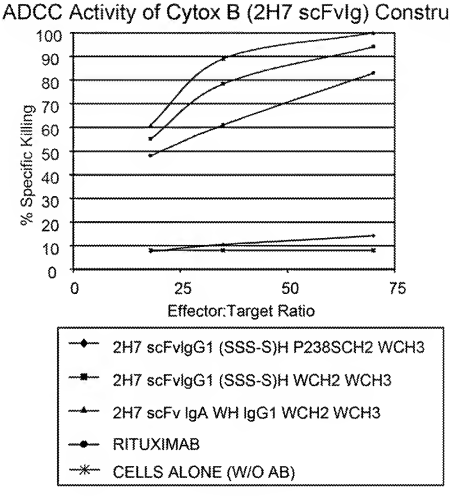

ADCC Activity of Cytox B (2H7 scFvIg) Constructs
% Specific Killing
100
90
80
70
60
50
40
30
20
10
0
0
25
50
75
Effector:Target Ratio
2H7 scFvIgG1 (SSS-S)H P238SCH2 WCH3
2H7 scFvIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA WH IgG1 WCH2 WCH3
RITUXIMAB
CELLS ALONE (W/O AB)

Fig. 13

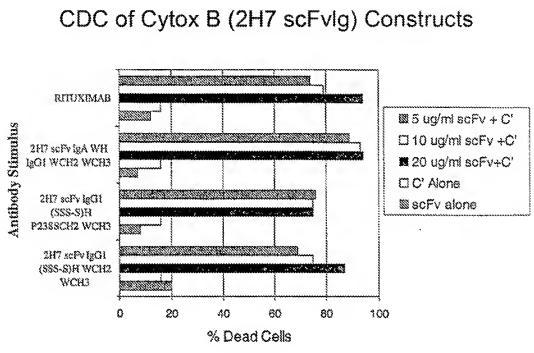
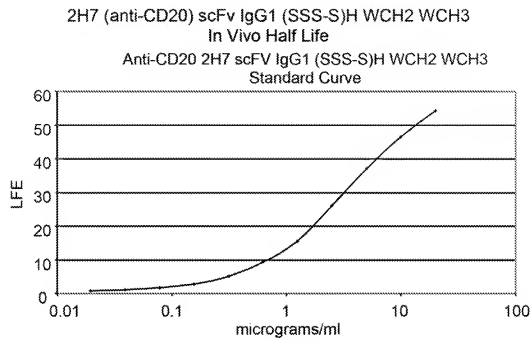

CDC of Cytox B (2H7 scFvIg) Constructs
RITUXIMAB
2H7 scFv IgA WH IgG1 WCH2 WCH3
2H7 scFv IgG1 (SSS-S)H P238SCH2 WCH3
2H7 scFv IgG1 (SSS-S)H WCH2 WCH3
Antibody Stimulus
0
20
40
60
80
100
% Dead Cells
5 ug/ml scFv + C'
10 ug/ml scFv +C'
20 ug/ml scFv+C'
C' Alone
scFv alone

Fig. 14



Macaque A99314

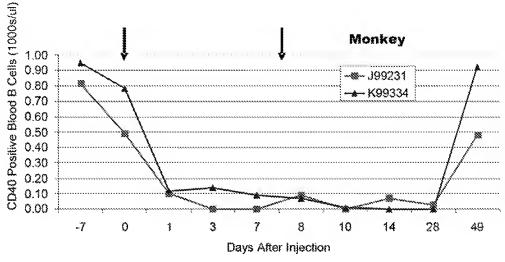
	Day	Binding intensity At 1:50 dilution of serum	estimated concentration (◊g/ml)
	–7	0.213	<0.1
Injection #1 →	0	0.227	<0.1
	1	7.79	25.1
	3	5.51	15.6
Injection #2 →	7	3.37	9.4
	8	11.33	41.7
	10	5.45	15.4
	14	0.27	<0.1

Macaque F98081

	Day	Binding intensity At 1:50 dilution of serum	estimated concentration (◊g/ml)
	–7	0.208	<0.1
Injection #1 →	0	0.219	<0.1
	1	6.73	21.9
	3	6.14	19.3
Injection #2 →	7	3.04	8.7
	8	9.83	33.8
	10	4.77	14.4
	14	0.231	<0.1

Fig. 15

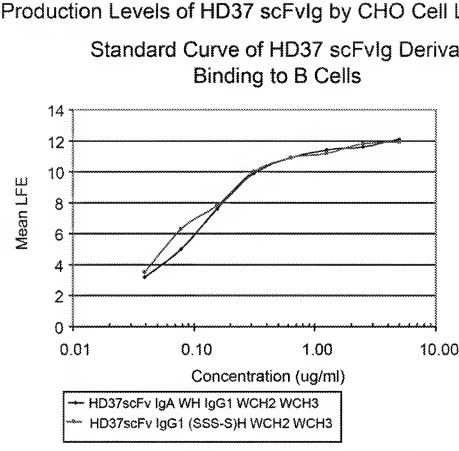
B Cell Depletion in macaques mediated by Cytox B20 (2H7 scFv IgG1 (SSS-S)H WCH2 WCH3) Construct



- CytoxB20 injections of 6mg/kg yields 3 week B-cell depletion
- 3-4 day half-life *in vivo*
- CD20 saturation in lymph node B-cells at d14
- No first dose effects
- No anti-chimeric antibody development

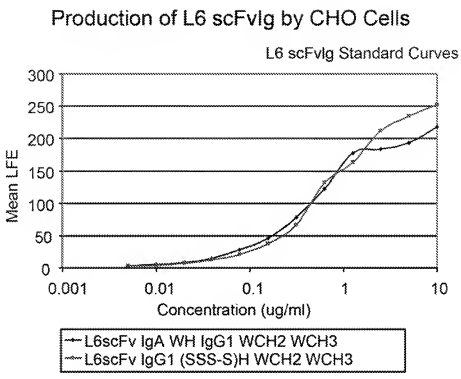
Fig. 16

Production Levels of HD37 scFvIg by CHO Cell Lines



Clone/Isolate	Mean LFE at 1:100	Estimated Concentration
BulkHD37 scFv IgA WH IgG1 WCH2 WCH3	11.2	> 60 ug/ml
1B2	10.4	>50 ug/ml
6C5	10.5	>50 ug/ml
4B1	8.6	>40 ug/ml
Bulk HD37 scFv IgG1 (SSS-S)HWCH2 WCH3	10.9	> 50 ug/ml
2G8	10.6	> 50 ug/ml
3F3	8.3	>40 ug/ml
3D9	11.1	> 60 ug/ml

Fig. 17



Construct	Mean LFE 1:20	Estimated Concentration
L6scFv IgA WH IgG1 WCH2 WCH3 unamplified CHO sup	51.1	6.25 ug/ml
L6scFv IgG1 (SSS-S)H WCH2 WCH3 unamplified CHO sup	23.0	3.2 ug/ml

Fig. 18

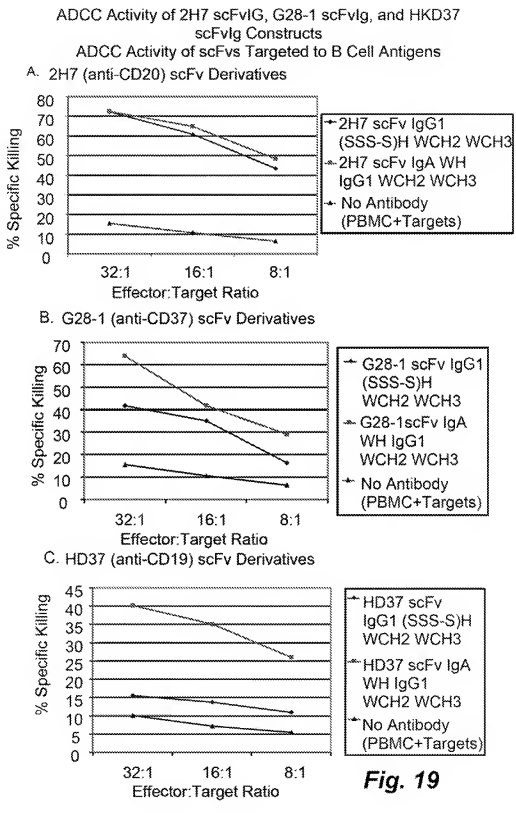

ADCC Activity of 2H7 scFvIG, G28-1 scFvIg, and HKD37 scFvIg Constructs
ADCC Activity of scFvs Targeted to B Cell Antigens
A. 2H7 (anti-CD20) scFv Derivatives
% Specific Killing
80
70
60
50
40
30
20
10
0
32:1
16:1
8:1
Effector:Target Ratio
2H7 scFv IgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA WH IgG1 WCH2 WCH3
No Antibody (PBMC+Targets)
B. G28-1 (anti-CD37) scFv Derivatives
% Specific Killing
70
60
50
40
30
20
10
0
32:1
16:1
8:1
Effector:Target Ratio
G28-1 scFv IgG1 (SSS-S)H WCH2 WCH3
G28-1scFv IgA WH IgG1 WCH2 WCH3
No Antibody (PBMC+Targets)
C. HD37 (anti-CD19) scFv Derivatives
% Specific Killing
45
40
35
30
25
20
15
10
5
0
32:1
16:1
8:1
Effector:Target Ratio
HD37 scFv IgG1 (SSS-S)H WCH2 WCH3
HD37 scFv IgA WH IgG1 WCH2 WCH3
No Antibody (PBMC+Targets)

Fig. 19

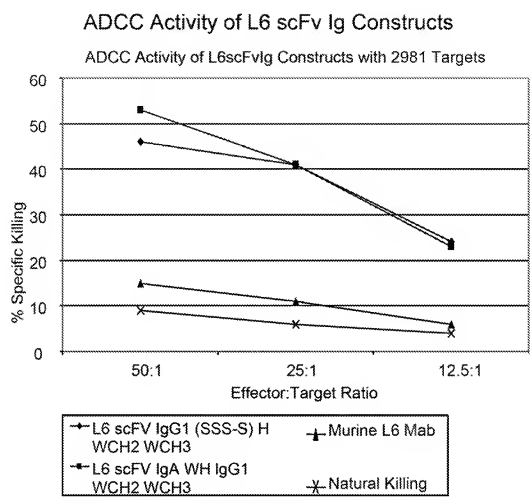

ADCC Activity of L6 scFv Ig Constructs
ADCC Activity of L6scFvIg Constructs with 2981 Targets
% Specific Killing
60
50
40
30
20
10
0
50:1
25:1
12.5:1
Effector:Target Ratio
L6 scFV IgG1 (SSS-S) H WCH2 WCH3
L6 scFV IgA WH IgG1 WCH2 WCH3
Murine L6 Mab
Natural Killing

Fig. 20

SDS-PAGE Analysis of L6 and 2H7
scFvIg Fusion Proteins.

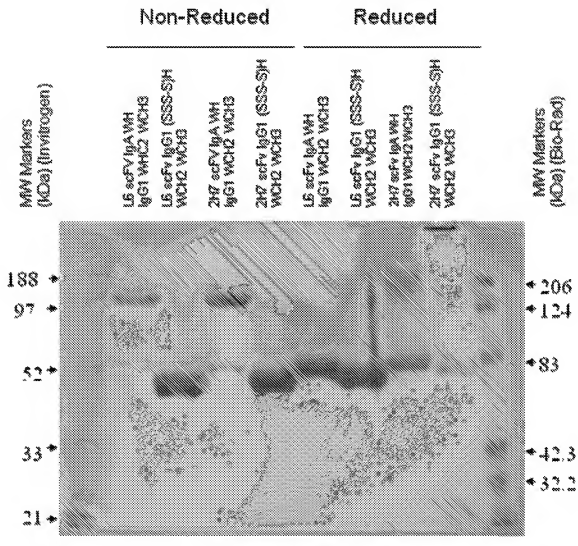


Fig. 21

SDS-PAGE Analysis of G28-1 and HD37
scFvIg Fusion Proteins.

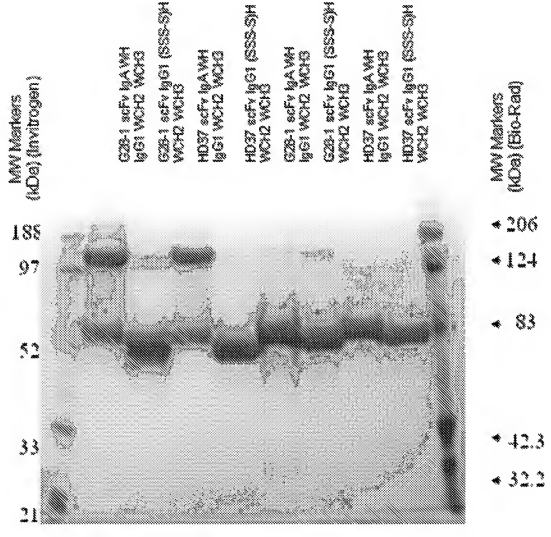


Fig. 22

Sequence alignment of human and llama Fc regions

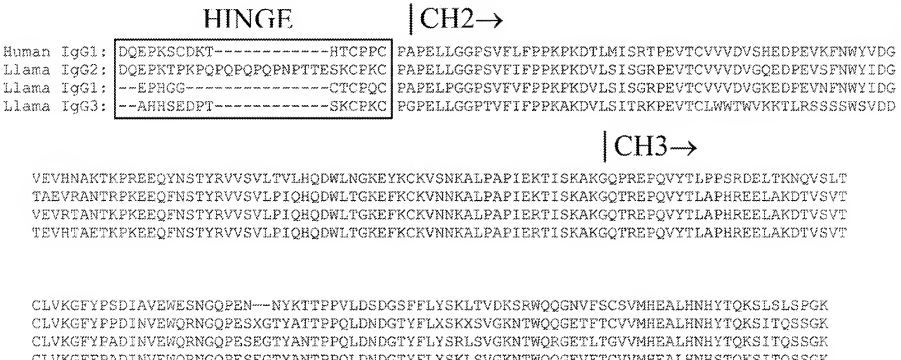


Fig. 23

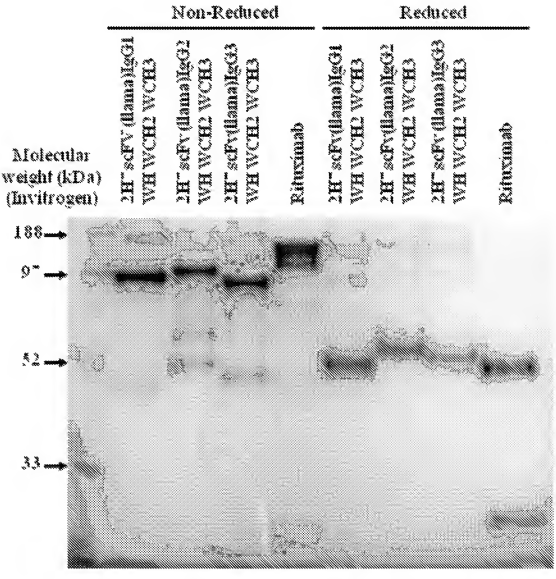

Non-Reduced
Reduced
Molecular weight (kDa) (Invitrogen)
2H7 scFv (llama)IgG1 WH WCH2 WCH3
2H7 scFv (llama)IgG2 WH WCH2 WCH3
2H7 scFv (llama)IgG3 WH WCH2 WCH3
Rituximab
188
97
52
33

Fig. 24

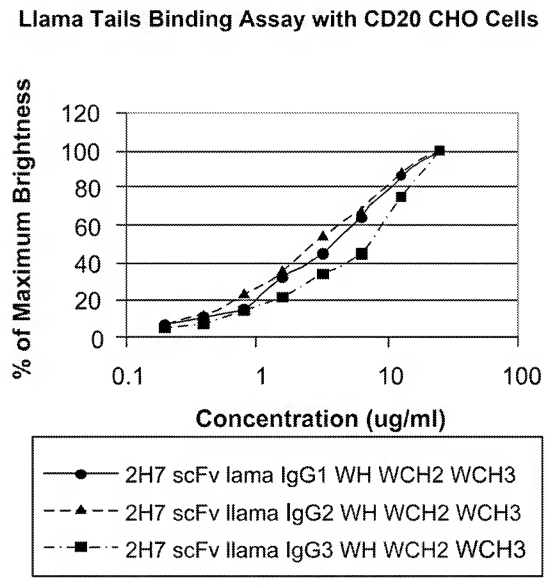

Llama Tails Binding Assay with CD20 CHO Cells
% of Maximum Brightness
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (ug/ml)
2H7 scFv lama IgG1 WH WCH2 WCH3
2H7 scFv llama IgG2 WH WCH2 WCH3
2H7 scFv llama IgG3 WH WCH2 WCH3

Fig. 25

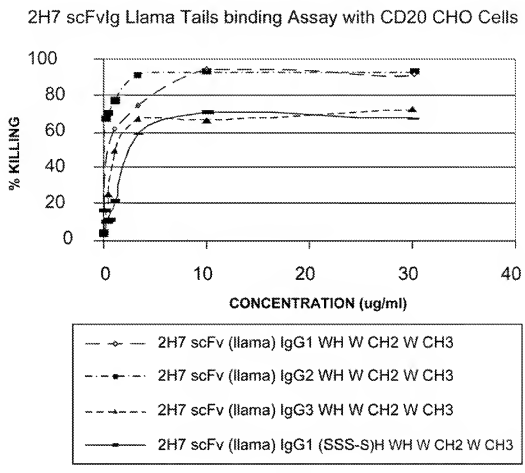

2H7 scFvIg Llama Tails binding Assay with CD20 CHO Cells
% KILLING
100
80
60
40
20
0
0
10
20
30
40
CONCENTRATION (ug/ml)
2H7 scFv (llama) IgG1 WH W CH2 W CH3
2H7 scFv (llama) IgG2 WH W CH2 W CH3
2H7 scFv (llama) IgG3 WH W CH2 W CH3
2H7 scFv (llama) IgG1 (SSS-S)H WH W CH2 W CH3

Fig. 26

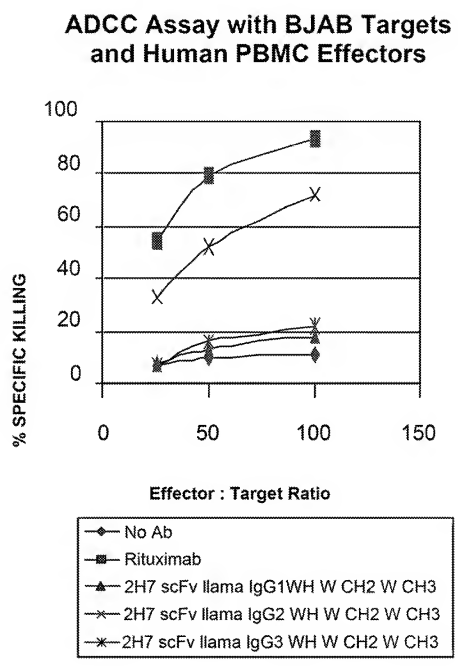

ADCC Assay with BJAB Targets and Human PBMC Effectors
% SPECIFIC KILLING
100
80
60
40
20
0
0
50
100
150
Effector : Target Ratio
No Ab
Rituximab
2H7 scFv llama IgG1WH W CH2 W CH3
2H7 scFv llama IgG2 WH W CH2 W CH3
2H7 scFv llama IgG3 WH W CH2 W CH3

Fig. 27

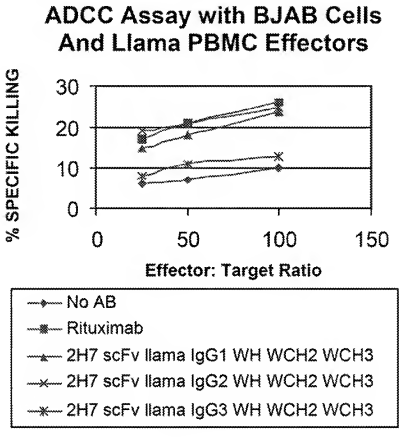

ADCC Assay with BJAB Cells
And Llama PBMC Effectors
% SPECIFIC KILLING
30
20
10
0
0
50
100
150
Effector: Target Ratio
No AB
Rituximab
2H7 scFv llama IgG1 WH WCH2 WCH3
2H7 scFv llama IgG2 WH WCH2 WCH3
2H7 scFv llama IgG3 WH WCH2 WCH3

Fig. 28

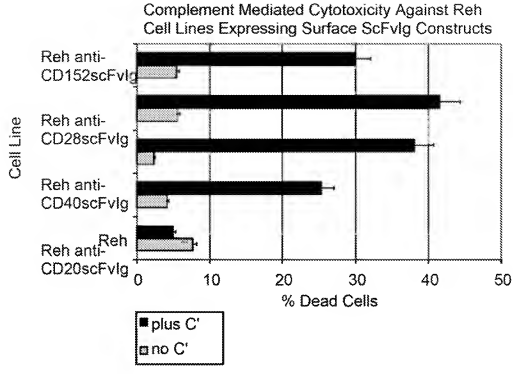

Complement Mediated Cytotoxicity Against Reh
Cell Lines Expressing Surface ScFvIg Constructs
Cell Line
Reh anti-CD152scFvIg
Reh anti-CD28scFvIg
Reh anti-CD40scFvIg
Reh anti-CD20scFvIg
Reh
0
10
20
30
40
50
% Dead Cells
plus C'
no C'

Fig. 29

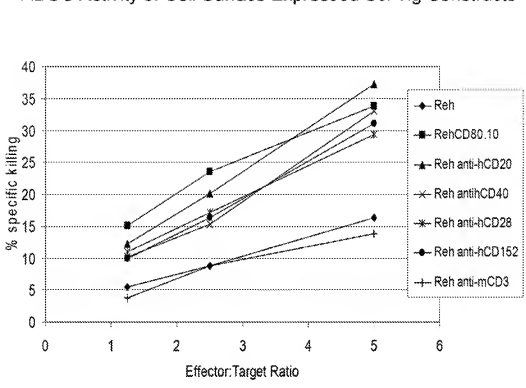

ADCC Activity of Cell Surface Expressed ScFvIg Constructs
% specific killing
Effector:Target Ratio
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5
6
Reh
RehCD80.10
Reh anti-hCD20
Reh antihCD40
Reh anti-hCD28
Reh anti-hCD152
Reh anti-mCD3

Fig. 30

Ig Constructs and Nomenclature:

Name Identifier	Hinge Sequence	CH2 Sequence	CH3 Sequence
hIgG1 (CCC-P)H WCH2 WCH3	IgG1 WT Hinge (CCC-P)	Wild Type CH2	Wild Type CH3
hIgG1 (SSS-S)H WCH2 WCH3	IgG1 Mutant Hinge (SSS-S)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)
VH L11S hIgG1 (SSS-S)H WCH2 WCH3	IgG1 Mutant Hinge (SSS-S)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)
IgG1 (SSC-S)H WCH2 WCH3	IgG1 Mutant Hinge (SSC-S)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)
IgG1 (SCS-S)H WCH2 WCH3	IgG1 Mutant Hinge (SCS-S)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)
IgG1 (CSS-S)H WCH2 WCH3	IgG1 Mutant Hinge (CSS-S)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)
IgG1 (SSS-S)H P238S CH2 WCH3	IgG1 Mutant Hinge (SSS)	Mutant CH2 (IgG1) Pro→Ser 238	Wild type CH3 (IgG1)
IgA WH hIgG1 WCH2 WCH3	IgA Hinge	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)
IgA WH IgA WCH2 WCH3	IgA Hinge	Wild type CH2 (IgA)	Wild type CH3 (IgA)
IgA WH IgA WCH2 T4CH3	IgA Hinge	Wild type CH2 (IgA)	Truncated CH3 (IgA) Missing 3 aa at COOH

Fig. 31

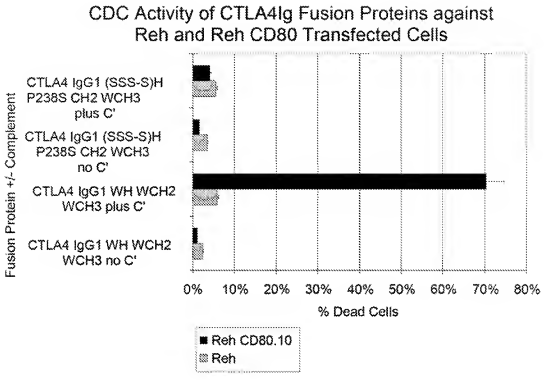

CDC Activity of CTLA4Ig Fusion Proteins against Reh and Reh CD80 Transfected Cells
Fusion Protein +/- Complement
CTLA4 IgG1 (SSS-S)H P238S CH2 WCH3 plus C'
CTLA4 IgG1 (SSS-S)H P238S CH2 WCH3 no C'
CTLA4 IgG1 WH WCH2 WCH3 plus C'
CTLA4 IgG1 WH WCH2 WCH3 no C'
0%
10%
20%
30%
40%
50%
60%
70%
80%
% Dead Cells
Reh CD80.10
Reh

Fig. 32

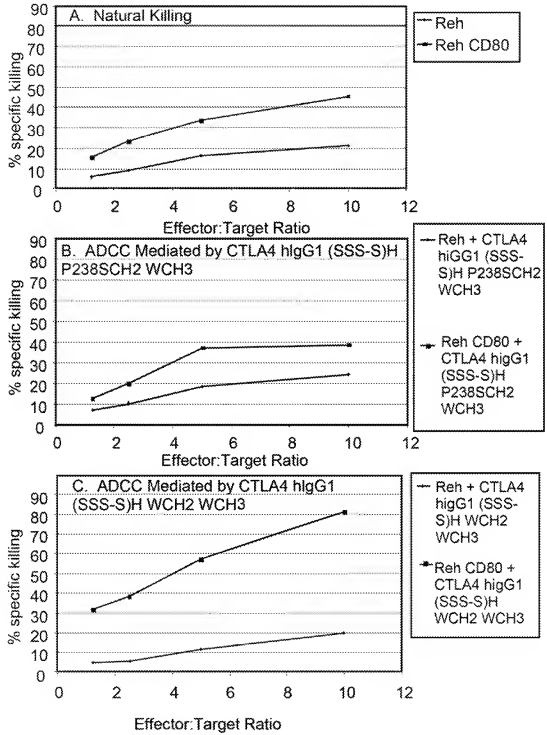

A. Natural Killing
Reh
Reh CD80
% specific killing
Effector:Target Ratio
B. ADCC Mediated by CTLA4 hIgG1 (SSS-S)H P238SCH2 WCH3
Reh + CTLA4 hiGG1 (SSS-S)H P238SCH2 WCH3
Reh CD80 + CTLA4 higG1 (SSS-S)H P238SCH2 WCH3
C. ADCC Mediated by CTLA4 hIgG1 (SSS-S)H WCH2 WCH3
Reh + CTLA4 higG1 (SSS-S)H WCH2 WCH3
Reh CD80 + CTLA4 higG1 (SSS-S)H WCH2 WCH3

Fig. 33

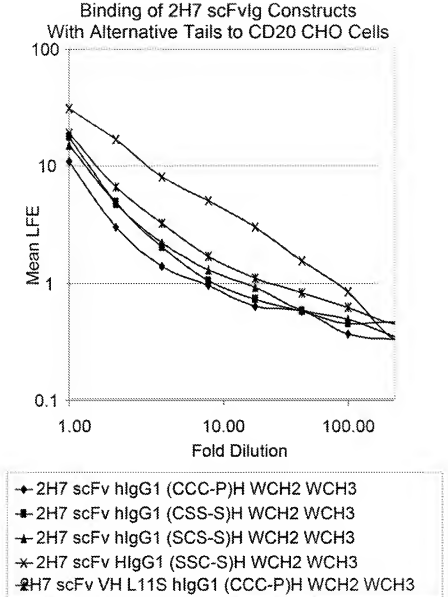

Binding of 2H7 scFvIg Constructs
With Alternative Tails to CD20 CHO Cells
100
10
1
0.1
Mean LFE
1.00
10.00
100.00
Fold Dilution
2H7 scFv hIgG1 (CCC-P)H WCH2 WCH3
2H7 scFv hIgG1 (CSS-S)H WCH2 WCH3
2H7 scFv hIgG1 (SCS-S)H WCH2 WCH3
2H7 scFv HIgG1 (SSC-S)H WCH2 WCH3
2H7 scFv VH L11S hIgG1 (CCC-P)H WCH2 WCH3

Fig. 34

Immunoblot Analysis of protein immunoprecipitates from COS transfections of 2H7 scFvIg Constructs

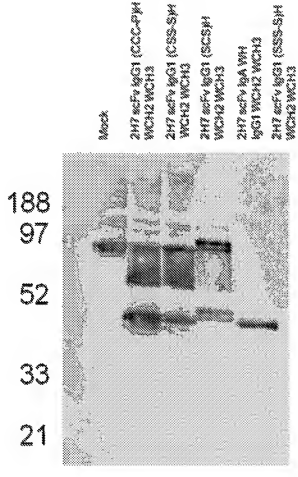


Fig. 35

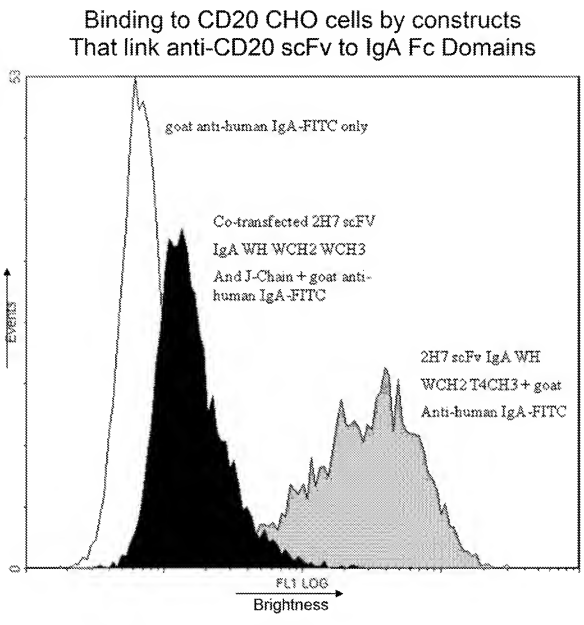

Binding to CD20 CHO cells by constructs
That link anti-CD20 scFv to IgA Fc Domains
63
0
EVENTS
goat anti-human IgA-FITC only
Co-transfected 2H7 scFV
IgA WH WCH2 WCH3
And J-Chain + goat anti-
human IgA-FITC
2H7 scFv IgA WH
WCH2 T4CH3 + goat
Anti-human IgA-FITC
FL1 LOG
Brightness

Fig. 36

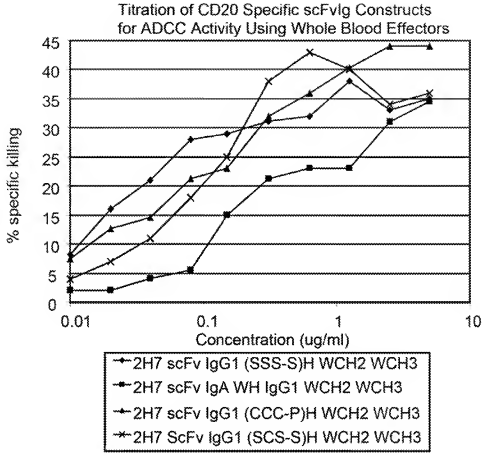

Titration of CD20 Specific scFvIg Constructs
for ADCC Activity Using Whole Blood Effectors
% specific killing
45
40
35
30
25
20
15
10
5
0
0.01
0.1
1
10
Concentration (ug/ml)
2H7 scFv IgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA WH IgG1 WCH2 WCH3
2H7 scFv IgG1 (CCC-P)H WCH2 WCH3
2H7 ScFv IgG1 (SCS-S)H WCH2 WCH3

Fig. 37

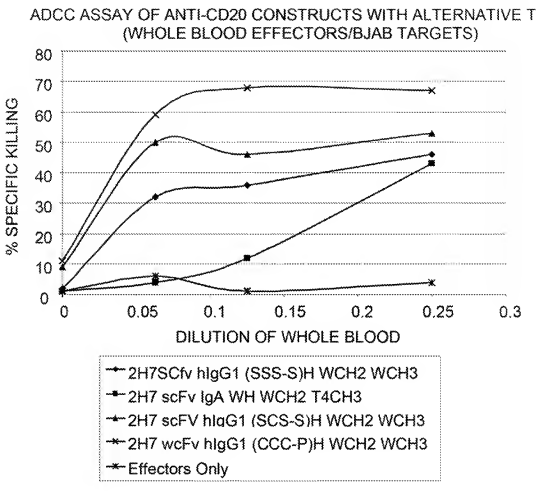

ADCC ASSAY OF ANTI-CD20 CONSTRUCTS WITH ALTERNATIVE TAILS
(WHOLE BLOOD EFFECTORS/BJAB TARGETS)
% SPECIFIC KILLING
80
70
60
50
40
30
20
10
0
0
0.05
0.1
0.15
0.2
0.25
0.3
DILUTION OF WHOLE BLOOD
2H7SCfv hIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA WH WCH2 T4CH3
2H7 scFV hIgG1 (SCS-S)H WCH2 WCH3
2H7 wcFv hIgG1 (CCC-P)H WCH2 WCH3
Effectors Only

Fig. 38

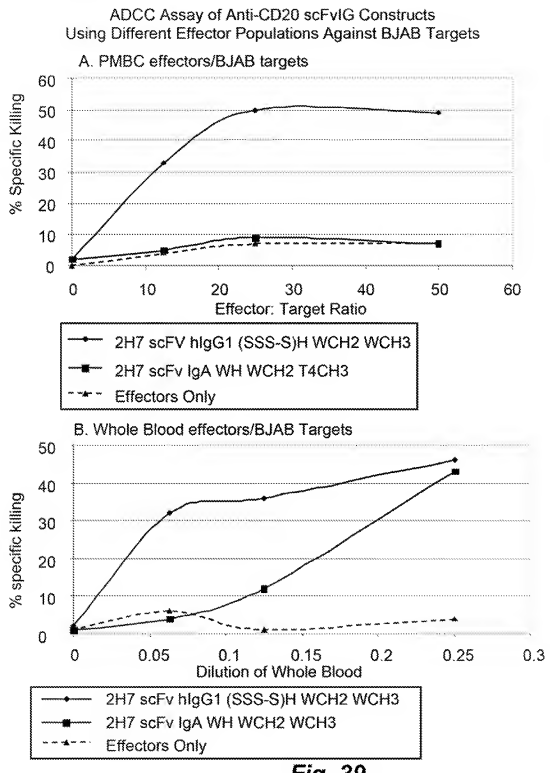

ADCC Assay of Anti-CD20 scFvIG Constructs
Using Different Effector Populations Against BJAB Targets
A. PMBC effectors/BJAB targets
% Specific Killing
0
10
20
30
40
50
60
Effector: Target Ratio
2H7 scFV hIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA WH WCH2 T4CH3
Effectors Only
B. Whole Blood effectors/BJAB Targets
% specific killing
0
10
20
30
40
50
0
0.05
0.1
0.15
0.2
0.25
0.3
Dilution of Whole Blood
2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv IgA WH WCH2 WCH3
Effectors Only

Fig. 39

Immunoblot of 2H7 scFv Ig constructs from COS Transfections (1 µl/well) compared to a Concentration Standard

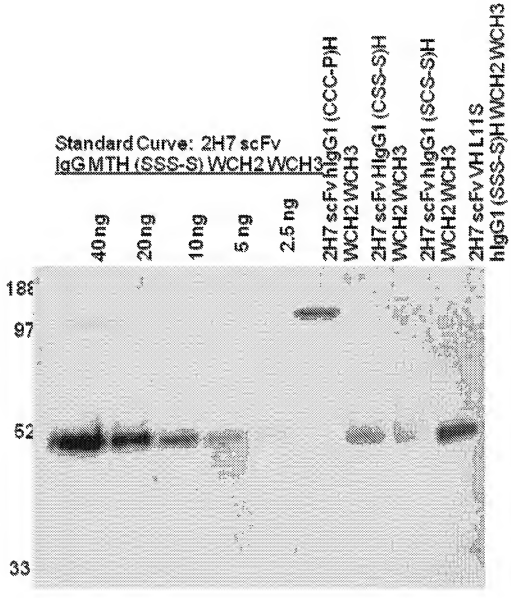


Fig. 40

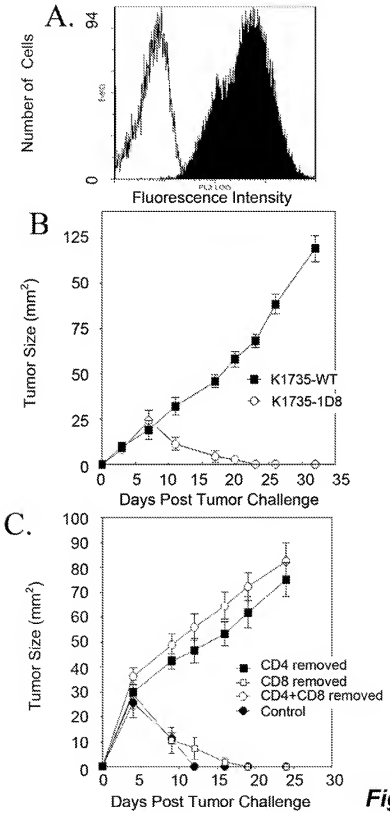

A.
Number of Cells
94
0
FL3 Log
Fluorescence Intensity
B
Tumor Size (mm²)
125
50
75
50
25
0
K1735-WT
K1735-1D8
0 5 10 15 20 25 30 35
Days Post Tumor Challenge
C.
Tumor Size (mm²)
100
90
80
70
60
50
40
30
20
10
0
CD4 removed
CD8 removed
CD4+CD8 removed
Control
0 5 10 15 20 25 30
Days Post Tumor Challenge

Fig. 41

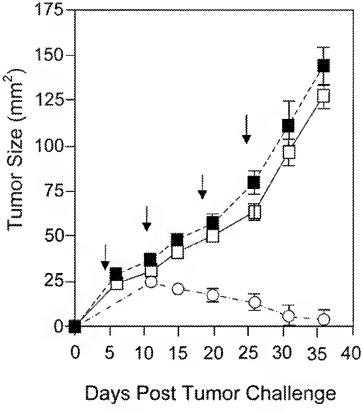

175
150
125
100
75
50
25
0
Tumor Size (mm^2)
0
5
10
15
20
25
30
35
40
Days Post Tumor Challenge

Fig. 42

Mixtures of K1735-WT and K1735-1D8 transfected tumor lines inhibit tumor outgrowth in C3H mice
tumor size (mm2)
days after immunization
0
20
40
60
80
100
120
140
160
0
5
10
15
20
25
30
35
K1735-WT 1446
K1735-WT 1422
K1735-WT 1447
K1735-WT 1420
K1735-WT 1421
K1735-WT+10%K1735-1D8 1492
K1735-WT+10%K1735-1D8 1699
K1735-WT+10%K1735-1D8 1493
K1735-WT+10%K1735-1D8 1496

Fig. 43

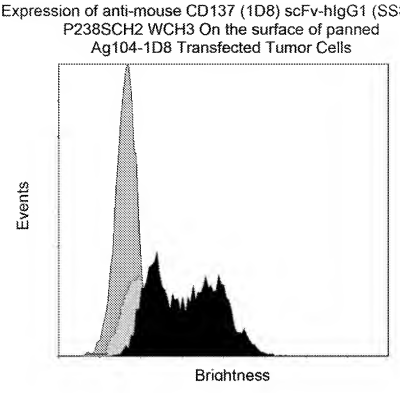

Expression of anti-mouse CD137 (1D8) scFv-hIgG1 (SSS-S)H
P238SCH2 WCH3 On the surface of panned
Ag104-1D8 Transfected Tumor Cells
Events
Brightness

Fig. 44

Coomassie Stained SDS-PAGE Gel of 2H7 scFv Ig Constructs

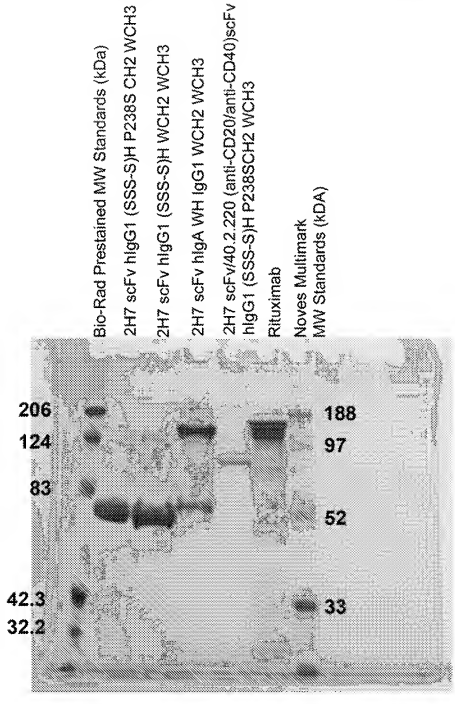


Fig. 45

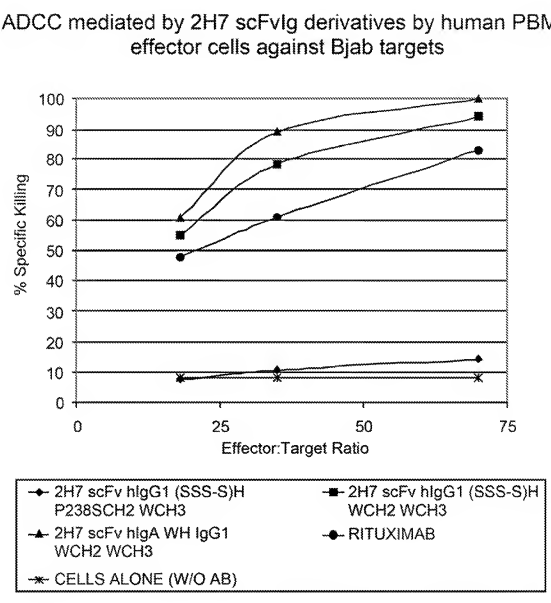
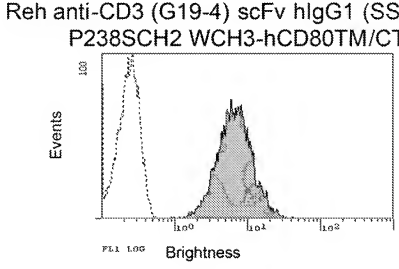

ADCC mediated by 2H7 scFvIg derivatives by human PBMC effector cells against Bjab targets
% Specific Killing
100
90
80
70
60
50
40
30
20
10
0
0
25
50
75
Effector:Target Ratio
2H7 scFv hIgG1 (SSS-S)H P238SCH2 WCH3
2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv hIgA WH IgG1 WCH2 WCH3
RITUXIMAB
CELLS ALONE (W/O AB)

Fig. 46

Cell surface expression of anti-human CD3 G19-4 scFv hIgG1 (SSS-S)H P238SCH2 WCH3-hCD80TM/CT on Reh and T51 Cells.



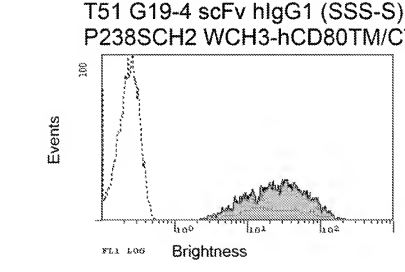


Fig. 47

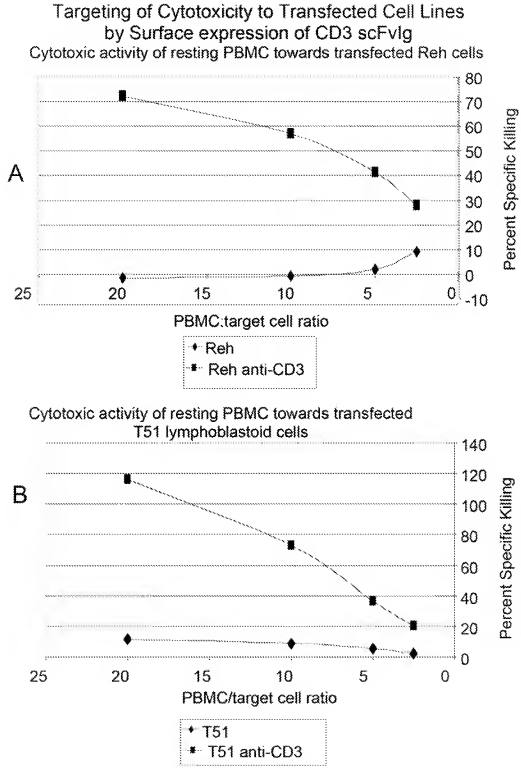

Targeting of Cytotoxicity to Transfected Cell Lines
by Surface expression of CD3 scFvIg
Cytotoxic activity of resting PBMC towards transfected Reh cells
A
80
70
60
50
40
30
20
10
0
-10
Percent Specific Killing
25
20
15
10
5
0
PBMC:target cell ratio
Reh
Reh anti-CD3
Cytotoxic activity of resting PBMC towards transfected
T51 lymphoblastoid cells
B
140
120
100
80
60
40
20
0
Percent Specific Killing
25
20
15
10
5
0
PBMC/target cell ratio
T51
T51 anti-CD3

Fig. 48

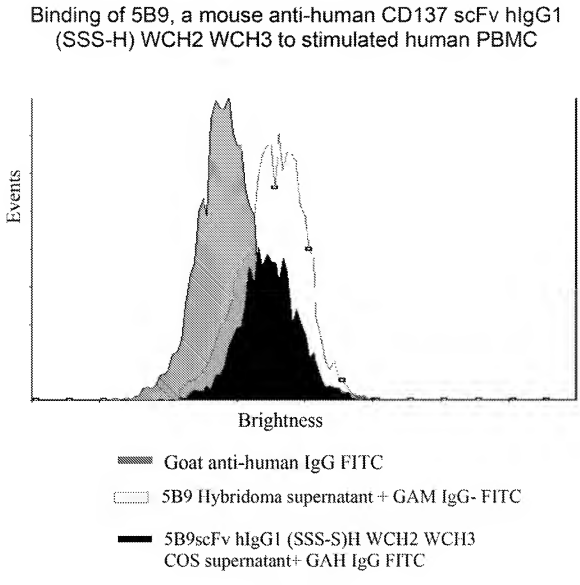
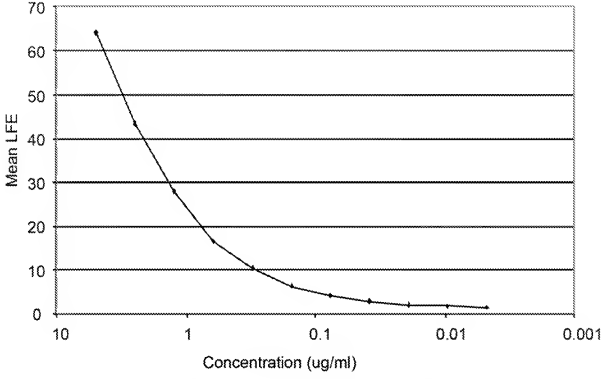

Binding of 5B9, a mouse anti-human CD137 scFv hIgG1 (SSS-H) WCH2 WCH3 to stimulated human PBMC
Events
Brightness
Goat anti-human IgG FITC
5B9 Hybridoma supernatant + GAM IgG- FITC
5B9scFv hIgG1 (SSS-S)H WCH2 WCH3 COS supernatant+ GAH IgG FITC

Fig. 49

Effect of V_HL11S Mutation on CytoxB20
2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3 Protein Expression

A. Standard Curve: 2H7VH-L11S-IgG1 (SSS-S)H WCH2 WCH3



B. CHO supernatant Brightness and Estimation of Protein concentrations from Standard Curve:

	CHO clone name				
	4F2	**4F5**	**3E5**	**6B11A**	**2B8A**
Mean LFE					
1/100	71.7	40.6	31.5	99.7	101.5
1/500	27.1	12.4	11.2	40.8	43
approx conc. µg/ml	**600**	**225**	**125**	**1000**	**1250**

Fig. 50

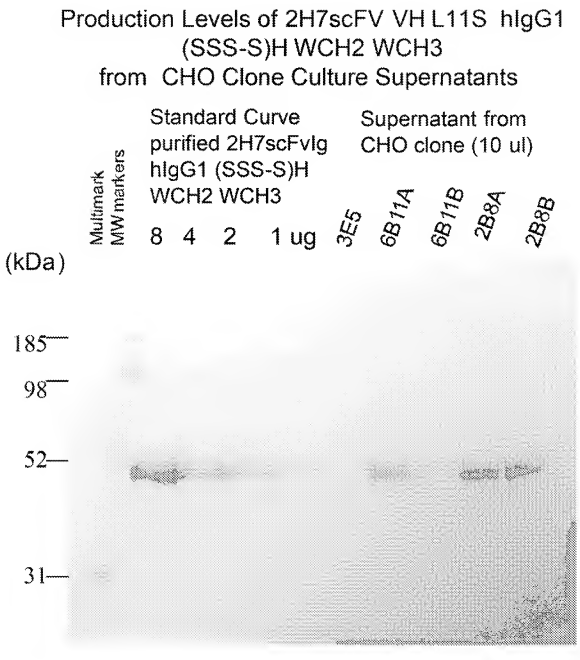

Production Levels of 2H7scFV VH L11S hIgG1
(SSS-S)H WCH2 WCH3
from CHO Clone Culture Supernatants
Standard Curve
purified 2H7scFvIg
hIgG1 (SSS-S)H
WCH2 WCH3
Supernatant from
CHO clone (10 ul)
Multimark
MW markers
8 4 2 1 ug
3E5
6B11A
6B11B
2B8A
2B8B
(kDa)
185
98
52
31

Fig. 51

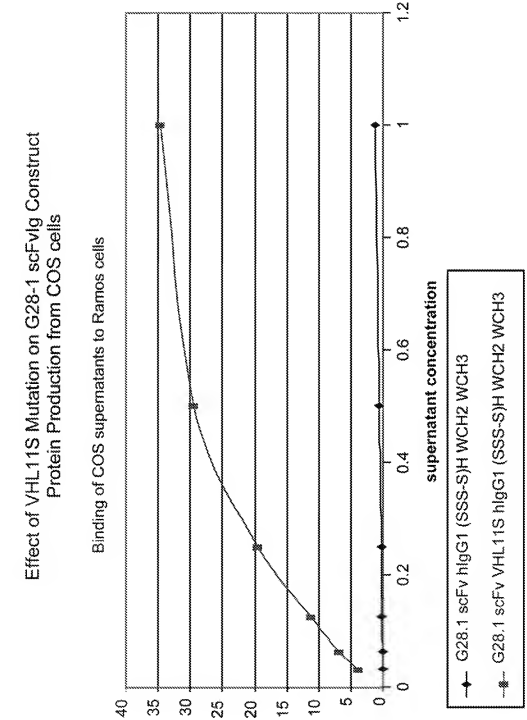

Effect of VHL11S Mutation on G28-1 scFvIg Construct
Protein Production from COS cells
Binding of COS supernatants to Ramos cells
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
supernatant concentration
G28.1 scFv hIgG1 (SSS-S)H WCH2 WCH3
G28.1 scFv VHL11S hIgG1 (SSS-S)H WCH2 WCH3

Fig. 52

Immunoblot of G28-1 scFvIg Derivatives

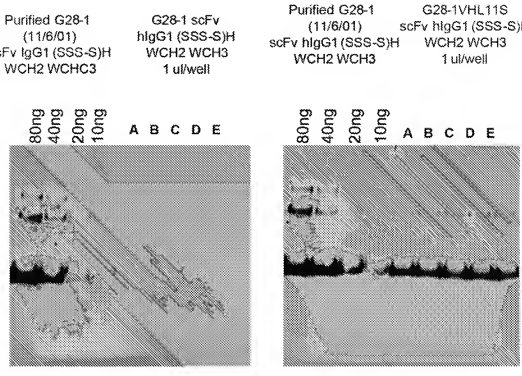


Fig. 53A ***Fig. 53B***

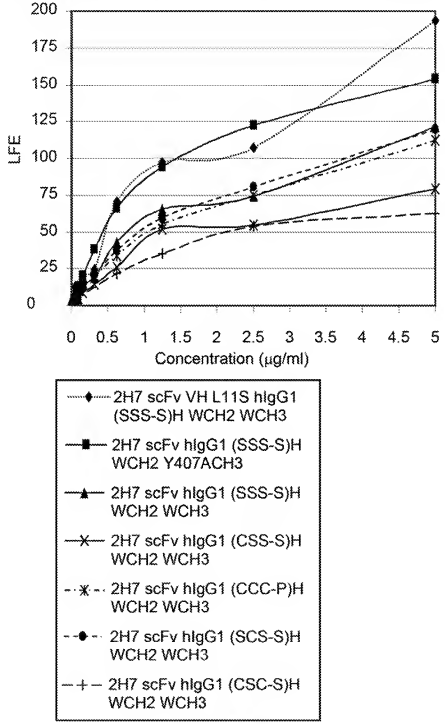

Binding of 2H7 scFvIg Constructs with Altered Hinges and CH3 domains to CD20 CHO Cells
LFE
200
175
150
125
100
75
50
25
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
Concentration (µg/ml)
2H7 scFv VH L11S hIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv hIgG1 (SSS-S)H WCH2 Y407ACH3
2H7 scFv hIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv hIgG1 (CSS-S)H WCH2 WCH3
2H7 scFv hIgG1 (CCC-P)H WCH2 WCH3
2H7 scFv hIgG1 (SCS-S)H WCH2 WCH3
2H7 scFv hIgG1 (CSC-S)H WCH2 WCH3

Fig. 54

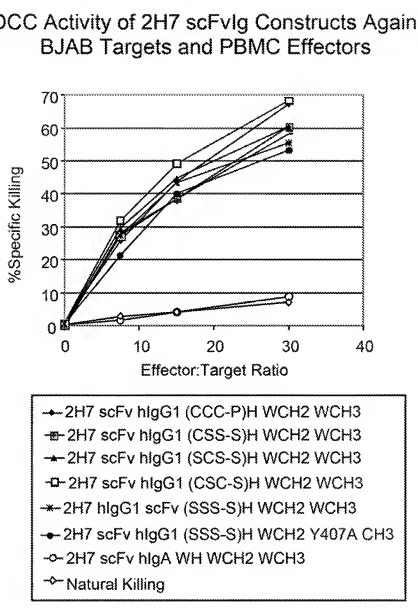

ADCC Activity of 2H7 scFvIg Constructs Against BJAB Targets and PBMC Effectors
%Specific Killing
70
60
50
40
30
20
10
0
0
10
20
30
40
Effector:Target Ratio
2H7 scFv hIgG1 (CCC-P)H WCH2 WCH3
2H7 scFv hIgG1 (CSS-S)H WCH2 WCH3
2H7 scFv hIgG1 (SCS-S)H WCH2 WCH3
2H7 scFv hIgG1 (CSC-S)H WCH2 WCH3
2H7 hIgG1 scFv (SSS-S)H WCH2 WCH3
2H7 scFv hIgG1 (SSS-S)H WCH2 Y407A CH3
2H7 scFv hIgA WH WCH2 WCH3
Natural Killing

Fig. 55

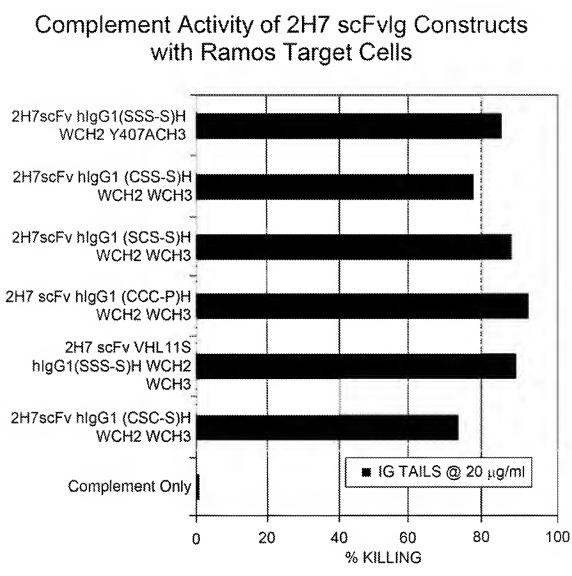

Complement Activity of 2H7 scFvIg Constructs with Ramos Target Cells
2H7scFv hIgG1(SSS-S)H WCH2 Y407ACH3
2H7scFv hIgG1 (CSS-S)H WCH2 WCH3
2H7scFv hIgG1 (SCS-S)H WCH2 WCH3
2H7 scFv hIgG1 (CCC-P)H WCH2 WCH3
2H7 scFv VHL11S hIgG1(SSS-S)H WCH2 WCH3
2H7scFv hIgG1 (CSC-S)H WCH2 WCH3
Complement Only
IG TAILS @ 20 μg/ml
0
20
40
60
80
100
% KILLING

Fig. 56

Binding of 2H7 scFvIg Derivatives to CD20CHO Cells

A. No fusion protein
B. 2H7 scFv hIgE CH2CH3CH4
C. 2H7 scFv hIgA WH WCH2 WCH3
D. 2H7 scFv hIgG (SSS-S)H WCH2 WCH3

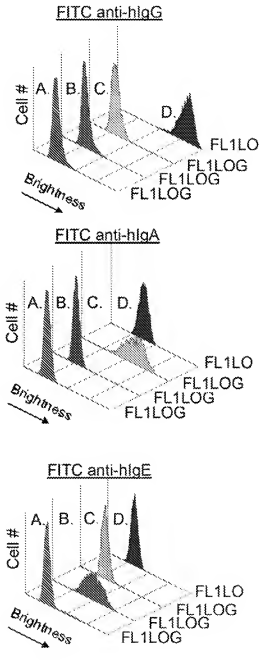
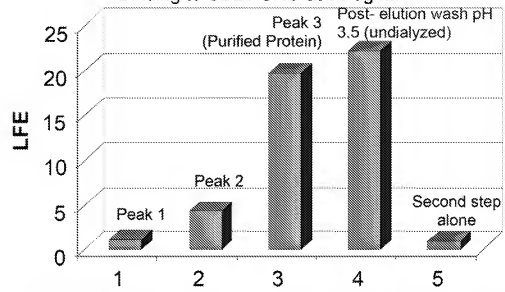
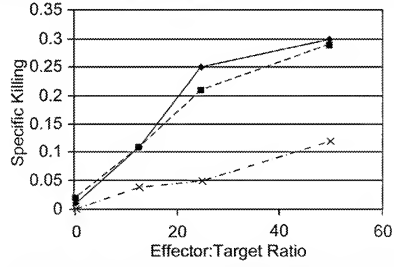


Fig. 57

A. 2H7 scFv VHL11S human IgE (WCH2 WCH3 WCH4) Binding to CD20 CHO at 30 ug/ml



B. ADCC Activity of 2H7 VHL11S IgE (WCH2 WCH3 WCH4) Protein Fractions with **PBMC** Effectors and Bjab Targets



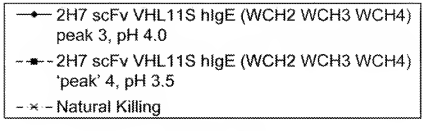


Fig. 58

Binding Data for COS derived α-CD20 (2H7) scFv VHL11S
mIg E (WCH2 WCH3 WCH4) and
mIgA (WH WCH2 WCH3) Tailed Molecules

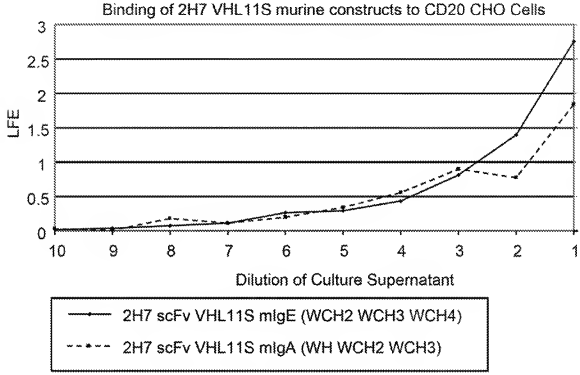


Fig. 59

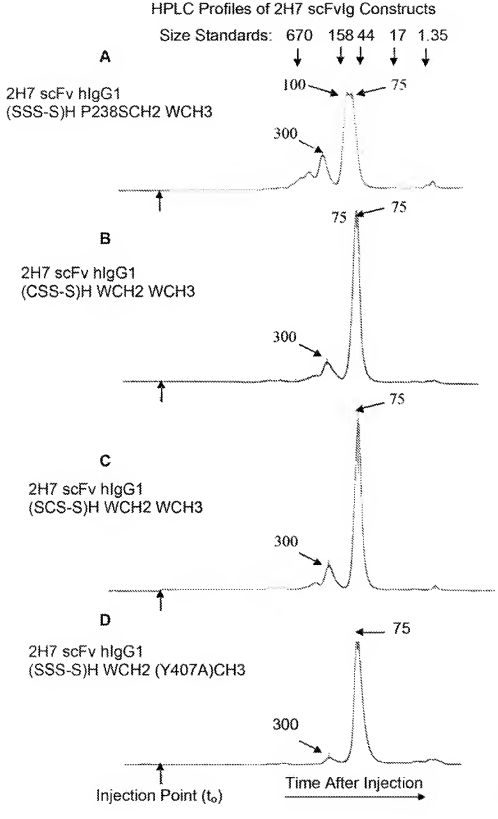

HPLC Profiles of 2H7 scFvIg Constructs
Size Standards: 670 158 44 17 1.35
A
2H7 scFv hIgG1
(SSS-S)H P238SCH2 WCH3
100
75
300
B
2H7 scFv hIgG1
(CSS-S)H WCH2 WCH3
75
75
300
C
2H7 scFv hIgG1
(SCS-S)H WCH2 WCH3
75
300
D
2H7 scFv hIgG1
(SSS-S)H WCH2 (Y407A)CH3
75
300
Injection Point (t_o)
Time After Injection

Fig. 60

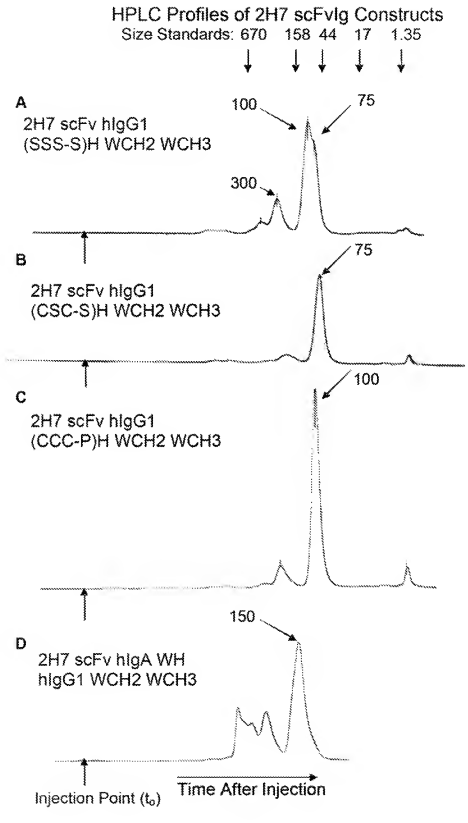

HPLC Profiles of 2H7 scFvIg Constructs
Size Standards: 670 158 44 17 1.35
A
2H7 scFv hIgG1
(SSS-S)H WCH2 WCH3
100
75
300
B
2H7 scFv hIgG1
(CSC-S)H WCH2 WCH3
75
C
2H7 scFv hIgG1
(CCC-P)H WCH2 WCH3
100
D
2H7 scFv hIgA WH
hIgG1 WCH2 WCH3
150
Injection Point (t_o)
Time After Injection

Fig. 61

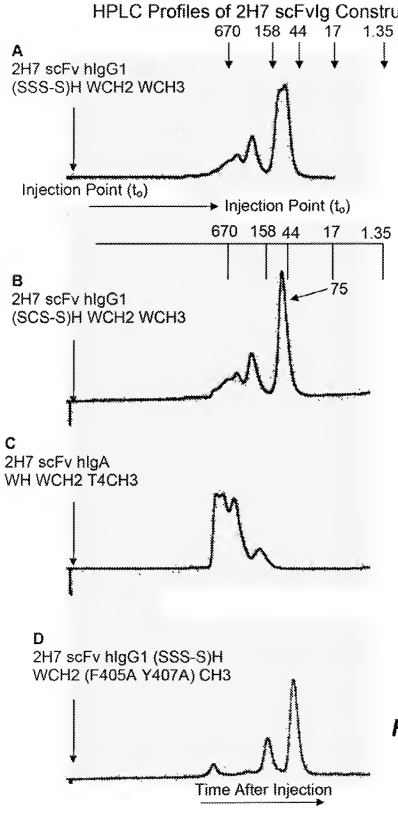

HPLC Profiles of 2H7 scFvIg Constructs
670 158 44 17 1.35
A
2H7 scFv hIgG1
(SSS-S)H WCH2 WCH3
Injection Point (t_o)
Injection Point (t_o)
670 158 44 17 1.35
B
2H7 scFv hIgG1
(SCS-S)H WCH2 WCH3
75
C
2H7 scFv hIgA
WH WCH2 T4CH3
D
2H7 scFv hIgG1 (SSS-S)H
WCH2 (F405A Y407A) CH3
Time After Injection

Fig. 62

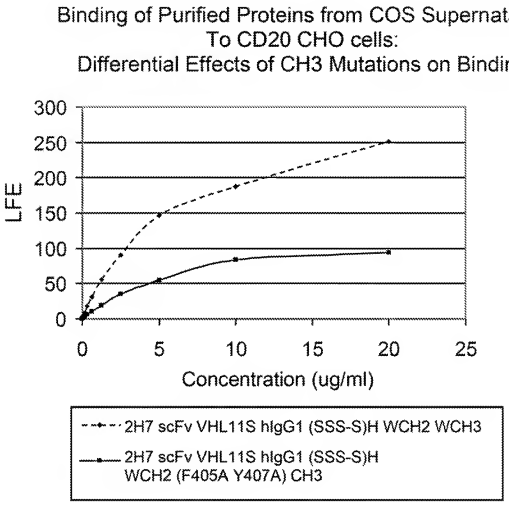

Binding of Purified Proteins from COS Supernatants
To CD20 CHO cells:
Differential Effects of CH3 Mutations on Binding
LFE
300
250
200
150
100
50
0
0
5
10
15
20
25
Concentration (ug/ml)
2H7 scFv VHL11S hIgG1 (SSS-S)H WCH2 WCH3
2H7 scFv VHL11S hIgG1 (SSS-S)H
WCH2 (F405A Y407A) CH3

Fig. 63

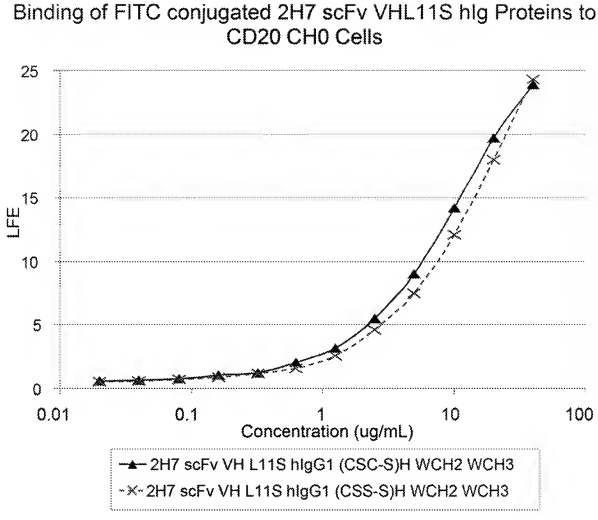

Binding of FITC conjugated 2H7 scFv VHL11S hIg Proteins to CD20 CH0 Cells
LFE
25
20
15
10
5
0
0.01
0.1
1
10
100
Concentration (ug/mL)
2H7 scFv VH L11S hIgG1 (CSC-S)H WCH2 WCH3
2H7 scFv VH L11S hIgG1 (CSS-S)H WCH2 WCH3

Fig. 64

Nonreducing SDS-PAGE on Protein A-Purified Lots of 2H7 scFv VHL11S hIg Constructs (10 ug/lane)

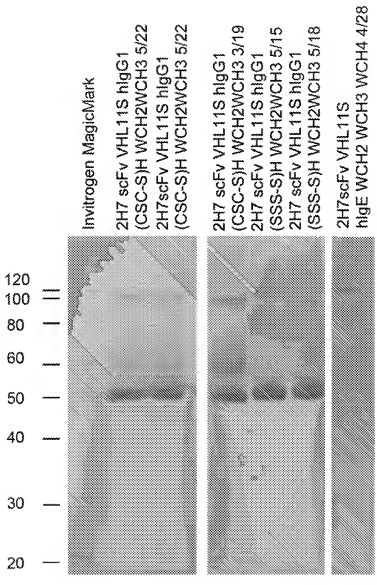


Fig. 65

Alterations in Human IgG Fc sequence
that differentially change effector function efficiency
P238
T256N
K290Q
K322S
P331S
A339P
hIgG2
hIgG3
hIgG4
hIgG1
rIgG2b
⊗ CDC residue
⊠ ADCC

Fig. 66

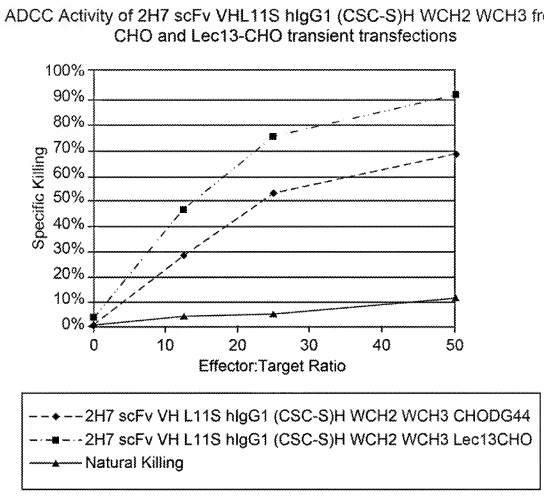

ADCC Activity of 2H7 scFv VHL11S hIgG1 (CSC-S)H WCH2 WCH3 from CHO and Lec13-CHO transient transfections
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Specific Killing
0
10
20
30
40
50
Effector:Target Ratio
2H7 scFv VH L11S hIgG1 (CSC-S)H WCH2 WCH3 CHODG44
2H7 scFv VH L11S hIgG1 (CSC-S)H WCH2 WCH3 Lec13CHO
Natural Killing

Fig. 67

CD16(ED) hIgG1 (SSS-S)H P238S CH2 WCH3 high and Low affinity alleles expressed as soluble molecules

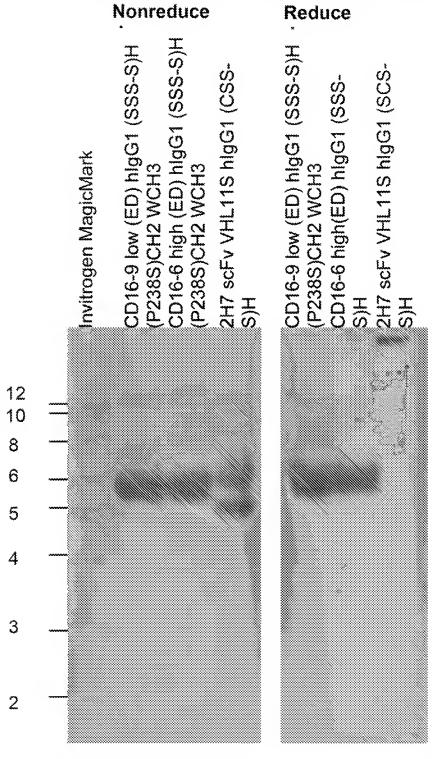


Fig. 68

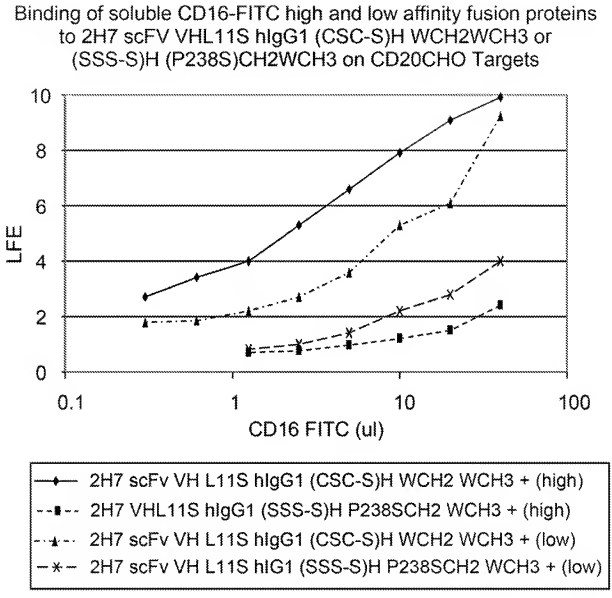
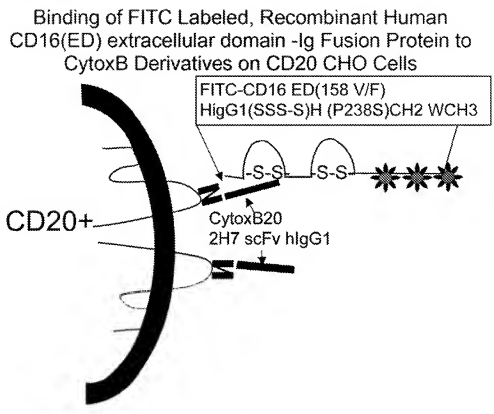

Binding of soluble CD16-FITC high and low affinity fusion proteins to 2H7 scFV VHL11S hIgG1 (CSC-S)H WCH2WCH3 or (SSS-S)H (P238S)CH2WCH3 on CD20CHO Targets
LFE
10
8
6
4
2
0
0.1
1
10
100
CD16 FITC (ul)
2H7 scFv VH L11S hIgG1 (CSC-S)H WCH2 WCH3 + (high)
2H7 VHL11S hIgG1 (SSS-S)H P238SCH2 WCH3 + (high)
2H7 scFv VH L11S hIgG1 (CSC-S)H WCH2 WCH3 + (low)
2H7 scFv VH L11S hIG1 (SSS-S)H P238SCH2 WCH3 + (low)

Fig. 69


Binding of FITC Labeled, Recombinant Human
CD16(ED) extracellular domain -Ig Fusion Protein to
CytoxB Derivatives on CD20 CHO Cells
FITC-CD16 ED(158 V/F)
HigG1(SSS-S)H (P238S)CH2 WCH3
-S-S-
-S-S-
CD20+
CytoxB20
2H7 scFv hIgG1

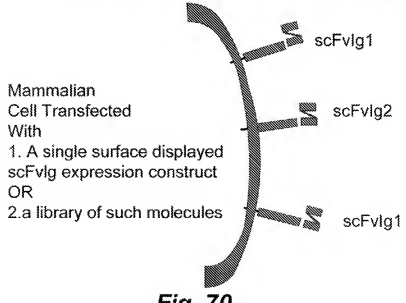

Expression of surface displayed SMIPs links
modified cDNAs with the altered fusion proteins
Mammalian
Cell Transfected
With
1. A single surface displayed
scFvIg expression construct
OR
2.a library of such molecules
scFvIg1
scFvIg2
scFvIg1

Fig. 70

CD37 mAbs and scFvIg Induce Apoptosis

	Bjab Staining	Annexin V Positive	
scFvIg	No scFvIg	17.5	
	2H7 MH	27	
	G28-1 MH	30.6	
	G28-1 IgAH	28.9	
	HD37 MH	29.1	
	(2H7+G28-1)MH	41	
	(2H7+HD37) MH	37.1	
	(G28-1+HD37) MH	35.3	
			plus GAM
mAbs	Ramos	AnnexinV Positive	AnnexinV positive
	cells alone	3	3.3
	2H7 Mab	1.4	3.1
	G28-1 Mab	18.3	8.7
	HD37 Mab	3.7	3.1
	G28-5	3.9	8.3
	2H7+G28-1	32.3	35.7
	2H7+HD37	5	10.5
	2H7+G28-5	5.7	19.4
	HD37+G28-1	26.9	50
	HD37+G28-5	8.2	18.4
	G28-1+G28-5	39.5	68.3

Fig. 71

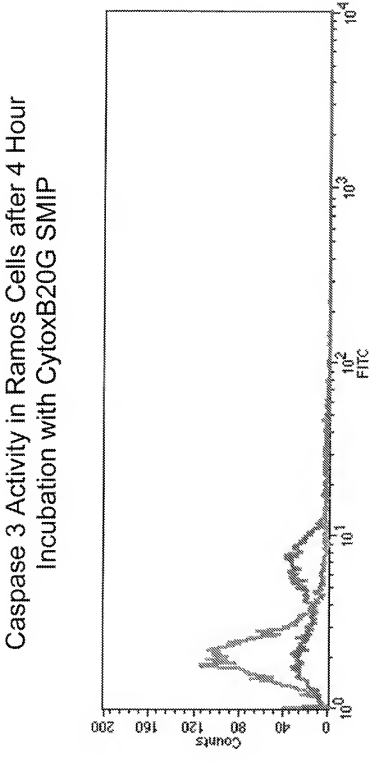

Caspase 3 Activity in Ramos Cells after 4 Hour Incubation with CytoxB20G SMIP
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FITC
media
CD20 SMIP

Fig. 72

Complement Dependent Cytotoxicity Mediated by CytoxB20G Derivatives
Treatment
MAB/SMIP at 5 ug/ml +C'
MAB/SMIP at 10 ug/ml +C'
MAB/SMIP at 20 ug/ml +C'
C' alone
Mab/SMIP alone
0
20
40
60
80
100
% killing
Rituximab
CytoxB20G P238S
Cytox B20G

Fig. 73

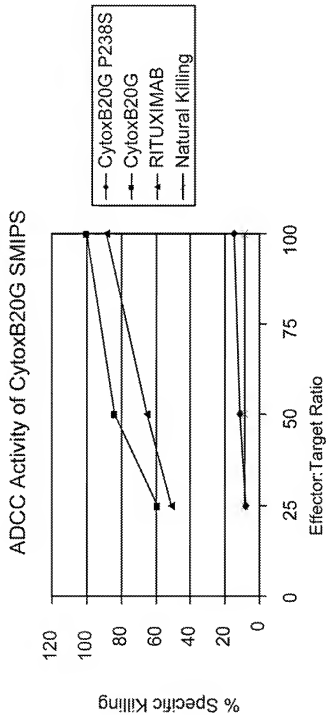

ADCC Activity of CytoxB20G SMIPS
% Specific Killing
120
100
80
60
40
20
0
0
25
50
75
100
Effector:Target Ratio
CytoxB20G P238S
CytoxB20G
RITUXIMAB
Natural Killing

Fig. 74

Binding of soluble FITC-CD16 to CytoxB20G on CD20 CHO Cells
MFI
12
10
8
6
4
2
0
0.1
1
10
100
Dilution of FITC-CD16
CytoxB20G
CytoxB20G P238S

Fig. 75

CytoxB20G and CytoxB20G P238S SMIPS bind to U937 Cells
Expressing FcγRI High Affinity FcR
MFI (x10-1)
2.5
2
1.5
1
0.5
0
0.001
0.01
0.1
1
10
100
Concentration (ug/ml)
CytoxB20G P238S
CytoxB20G

Fig. 76

B Cell Depletion Mediated by CytoxB20G SMIPs
% B Cells Versus Maximum
100
90
80
70
60
50
40
30
20
10
0
-7
3
13
23
33
43
Days After Injection
Cytox B20G J99231
Cytox B20G P238S F98081
Cytox B20G K99334
Cytox B20G P238S F99314

Fig. 77

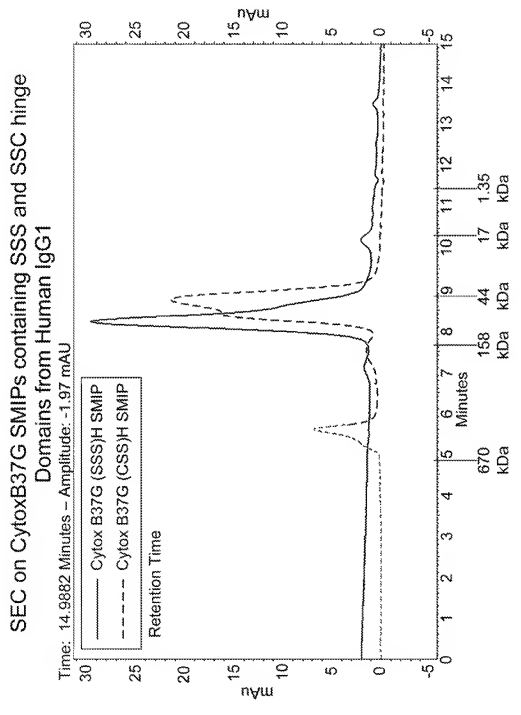

SEC on CytoxB37G SMIPs containing SSS and SSC hinge Domains from Human IgG1
Time: 14.9882 Minutes – Amplitude: -1.97 mAU
Cytox B37G (SSS)H SMIP
Cytox B37G (CSS)H SMIP
Retention Time
mAu
Minutes
670 kDa
158 kDa
44 kDa
17 kDa
1.35 kDa

Fig. 78

Binding of CytoxB37G SMIPs to B Cell Lymphoma Cell Lines

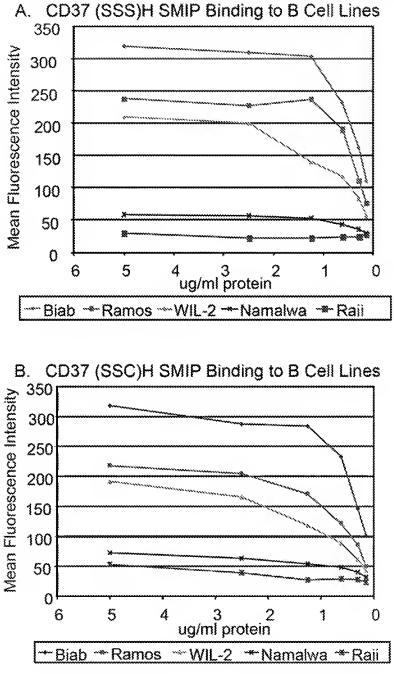


Fig. 79

AnnexinV-PI Staining of Ramos Cells Incubated 24 hours with CD37 SMIPS

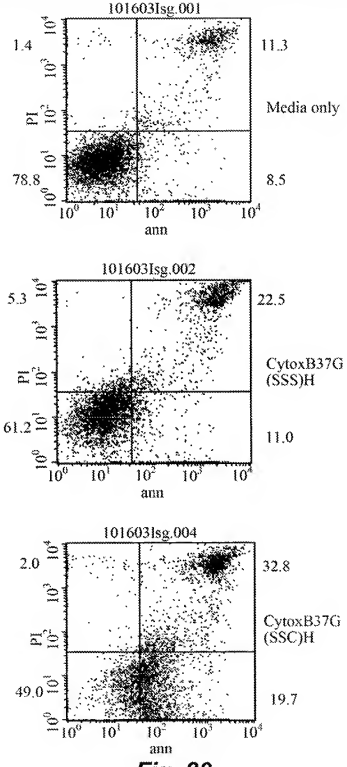


Fig. 80

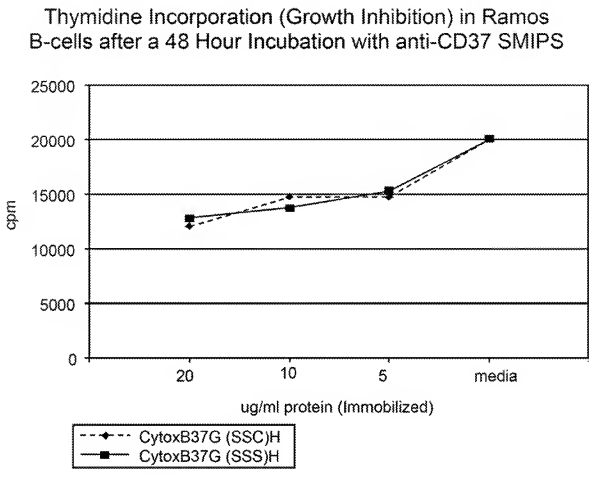

Thymidine Incorporation (Growth Inhibition) in Ramos B-cells after a 48 Hour Incubation with anti-CD37 SMIPS
25000
20000
15000
10000
5000
0
cpm
20
10
5
media
ug/ml protein (Immobilized)
CytoxB37G (SSC)H
CytoxB37G (SSS)H

Fig. 81

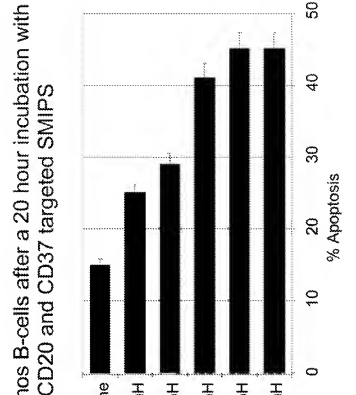

The Induction of Apoptosis in Ramos B-cells after a 20 hour incubation with different combinations of CD20 and CD37 targeted SMIPS
SMIP
Media Alone
CytoxB20G (CSS)H
Cytox B37G (CSS)H
Cytox B37G (SSS)H
CytoxB20G (CSS)H + CytoxB37G (CSS)H
CytoxB20G (CSS)H + CytoxB37G (SSS)H
0
10
20
30
40
50
% Apoptosis

Fig. 82

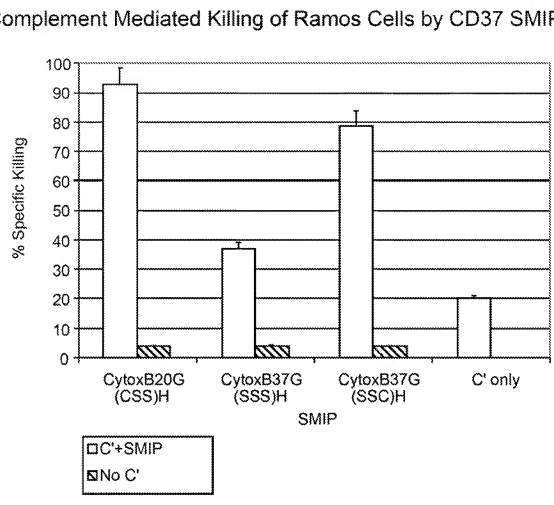

Complement Mediated Killing of Ramos Cells by CD37 SMIPs
% Specific Killing
100
90
80
70
60
50
40
30
20
10
0
CytoxB20G (CSS)H
CytoxB37G (SSS)H
CytoxB37G (SSC)H
C' only
SMIP
C'+SMIP
No C'

Fig. 83

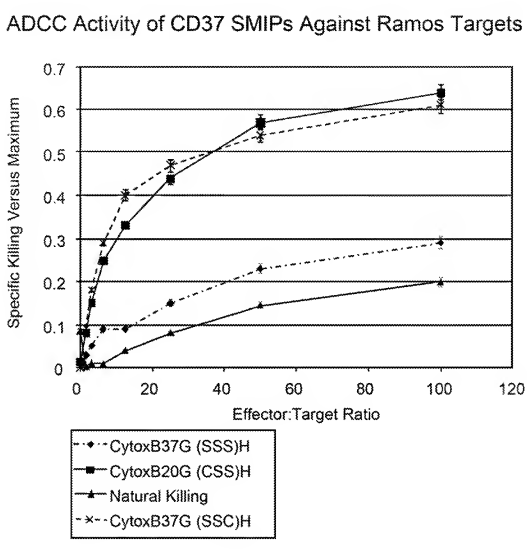

ADCC Activity of CD37 SMIPs Against Ramos Targets
Specific Killing Versus Maximum
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Effector:Target Ratio
CytoxB37G (SSS)H
CytoxB20G (CSS)H
Natural Killing
CytoxB37G (SSC)H

Fig. 84